ULTRASOUND CHARACTERIZATION OF ANTRAL FOLLICLE DYNAMICS IN POLYCYSTIC OVARY SYNDROME

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Polycystic ovary syndrome (PCOS) is the leading cause of anovulatory infertility in women. Disordered folliculogenesis in PCOS has long been characterized by three phenomena: (1) the accumulation of small follicles, (2) inhibition of follicular maturation, and (3) absence of follicular turnover. These defects presumably prevent ovulation, but have never been explored *in vivo*. An accurate understanding of antral follicle development in PCOS is critical to facilitate the diagnosis and identify targets for nutritional and pharmacologic interventions aimed at reinstating ovulation. The central objective of this dissertation was to characterize antral folliculogenesis in women with PCOS. In **Part 1**, we used serial ultrasonography to assess follicle growth and regression during a 4–5-week interval. Anovulatory cycles were considered in **Chapter 1**. We documented active components of follicle development amidst follicular excess in PCOS, and revealed that follicles become arrested at the mid-antral stage, but turnover more frequently than in normal ovaries. Sporadic ovulatory cycles were considered in **Chapter 2**. We observed earlier selection in women with PCOS than in regular ovulatory cycles and identified potential relationships between milder reproductive features and likelihood of sporadic ovulation. In **Part 2**, we began to explore the implications of disordered antral folliculogenesis on the diagnosis of PCOS and treatment of anovulation. The clinical utility of the sonographic criteria for PCOS were considered across the menstrual cycle in **Chapter 3**. We demonstrated that diagnostic markers of follicular excess are robust over time irrespective of cycle phase. Nutritional therapies for anovulation were reviewed in **Chapter 4**. We described the limitations of previous studies and identified opportunities for future research in the field. Collectively, this dissertation integrated ultrasonographic approaches from basic science to inform and improve the clinical management of women with PCOS.

BIOGRAPHICAL SKETCH

Brittany Yasemin Jarrett was born on April 4, 1990 to Cicek and Charles Jarrett. She grew up in Columbus, OH and was interested in learning and science from an early age. Over time, her interests evolved from marine biology to psychology, chemistry, and medicine. Her ultimate interest in nutrition brought her to Cornell University, where she completed the undergraduate Didactic Program in Dietetics and earned a Bachelor of Science degree in Nutritional Sciences (2008–2012). In her sophomore year, Brittany joined Dr. Marla Lujan's research group. Her goal was to learn more about the scientific method and clinical research. Little did she know, she would discover her passion for women's health and find a home for the next eight years. She gained expertise in ultrasound image analysis and completed an honors thesis on the use of automated techniques to evaluate ovarian morphology. This inspired her research interests and led her to apply to the Combined PhD/RD Program at Cornell (2012). She remained in Dr. Lujan's research group and began to investigate the mechanisms of ovulatory dysfunction in overweight women with polycystic ovary syndrome. She also designed and managed a four-month hypocaloric diet intervention aimed at reinstating ovulation in this population. Simultaneously with her doctorate, Brittany completed Cornell's dietetic internship (2014), the WHO/Cochrane/Cornell Summer Institute for Systematic Reviews in Nutrition for Global Policy Making (2016), and a translational field experience with the Academy of Nutrition and Dietetics' Evidence Analysis Library (2017). In her free time, Brittany relaxed by running, doing barre or yoga, and learning to bake.

This dissertation is dedicated to my mom, Cicek.

WE did it.

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PREFACE

Polycystic ovary syndrome (PCOS) affects one in ten women of reproductive age and is the leading cause of ovulatory dysfunction globally.[1,2] The condition is complex and influenced by a variety of factors, including age, ethnicity, genetics, and environment.[3] PCOS imparts significant consequences for *reproductive* (anovulation, androgen excess, infertility, pregnancy-related risks), *metabolic* (obesity, insulin resistance, type 2 diabetes mellitus, metabolic syndrome, cardiovascular disease), and *psychological (anxiety, depression, poor quality of life)* health across the lifespan.[4] Reproductive disturbances are often the best-recognized features and are consequently relied on to diagnose the condition and guide the selection of treatments that can address infertility.[5]

Diagnosis of PCOS

Diagnosis of PCOS is largely based on the Rotterdam criteria[6,7] and the presence of two of three cardinal features: oligo- or anovulation, androgen excess, and polycystic ovaries.[8,9] Other disorders that cause ovulatory dysfunction or androgen excess (e.g. thyroid abnormalities, hyperprolactinemia, nonclassical congenital adrenal hyperplasia) are excluded.[7] PCOS can be categorized into four different phenotypes based on the possible combinations of the cardinal features: (A) *Frank* (oligo- or anovulation, androgen excess, polycystic ovaries), (B) *Non-PCO* (oligo- or anovulation, androgen excess, normal ovaries), (C) *Ovulatory* (regular ovulatory cycles, androgen excess, polycystic ovaries), and (D) *Normoandrogenic* (oligo- or anovulation, normal androgen status, polycystic ovaries) (Table P.1).[8,9]

PCOS exists on a spectrum and varies in prevalence and severity among the four phenotypes. The hyperandrogenic phenotypes (Table P.1, Columns A–C) are the most common in consecutive patient and unselected populations[10–12] and represent the severest end of the spectrum. Androgen excess is positively associated with degree of reproductive dysfunction[10–12]

and confers increased risk for chronic metabolic diseases.[13] The normoandrogenic phenotype (Table P.1, Column D) is less common.[10–12] Inclusion of this variant is controversial,[14,15] because androgen excess has been considered a cornerstone of the syndrome since its first description in 1935.[14–17] However, multiple studies have shown that the combination of oligo- or anovulation and polycystic ovaries is associated with mild reproductive and endocrine features of PCOS.[10–12] Women with Normoandrogenic PCOS may have decreased risk for chronic metabolic disease, but further studies are needed to corroborate these data.[13,18]

Table P.1. PCOS phenotypes enabled by the Rotterdam criteria

	Frank (A)	Non-PCO (B)	Ovulatory (C)	Mild (D)
Oligo- or anovulation	Yes	Yes	No	Yes
Androgen excess	Yes	Yes	Yes	No
Polycystic ovaries	Yes	No	Yes	Yes
Prevalence[10]	66%	9%	13%	11%

Abbreviations: PCOS, polycystic ovary syndrome; PCO, polycystic ovaries.

Assessment of the cardinal features of PCOS is challenging.[18] The features are treated as dichotomous variables, but the optimal analytical technique is controversial and diagnostic thresholds remain largely undefined.[4,6,7,18] Consequently, clinicians and researchers rely on tools readily available at their sites and often establish thresholds based on internal normative ranges for each marker. Multiple professional societies have responded to these challenges with general recommendations for the assessment and definition of each feature.[7–9,14,15,19] Generally, oligo- or anovulation is judged by self-report of irregular menstrual cycles and confirmed in cases of eumenorrhea with biochemical evidence of anovulation (e.g. low serum progesterone during the suspected luteal phase).[7–9,14,15] Clinical or biochemical androgen excess is evaluated by physical examination of male-patterned hair growth (termed hirsutism) or elevated serum androgens (e.g.

total testosterone).[7–9,14,15] Polycystic ovaries are identified on ultrasonography by increased follicle number and/or ovarian size.[8,9,19,20]

Normal Ovarian Folliculogenesis

This dissertation focused on the mechanism of anovulation in PCOS. An overview of the normal process of ovarian folliculogenesis is necessary to understand the existing evidence and rationale for these studies. Broadly, ovarian follicles are cellular structures that contain immature oocytes, and folliculogenesis is defined as the process of follicular growth and atresia within the ovaries.[21,22] Proper follicle development relies on signals from the hypothalamus, pituitary, and ovary and is essential for the maturation of a healthy gamete and baby.[22,23]

Hypothalamic-Pituitary-Ovarian Axis

The hypothalamic-pituitary-ovarian axis coordinates female reproductive function.[23] The hormones produced by the anterior pituitary and/or ovaries provide feedback at the level of the hypothalamus and pituitary to regulate hormone production during the menstrual cycle.

Gonadotropin-releasing hormone (GnRH) is a peptide hormone synthesized and released from neurons in the hypothalamus. GnRH is released in a pulsatile manner into the pituitary portal network.[24] At the anterior pituitary, the hormone stimulates the synthesis and release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These processes are controlled by the size and frequency of GnRH pulses.[24] Slow GnRH pulse frequencies promote FSH synthesis, whereas fast GnRH pulse frequencies promote LH synthesis.[25,26]

FSH and LH are glycoprotein hormones produced in the gonotrophic cells of the anterior pituitary.[23] At the ovary, LH binds to LH receptors located on the membrane of follicular thecal cells, granulosa cells at later stages of development (i.e. ≥10 mm), and luteinized theca and granulosa cells of the corpus luteum (CL).[27] LH binds to theca cells at all stages of development

to stimulate ovarian androgen production (e.g. testosterone).[28] LH binds to granulosa cells of larger follicles to stimulate granulosa cell proliferation, growth, and estradiol production.[21,23,29] Luteinized cells of the CL also stimulate progesterone production during the luteal phase.[30] By contrast, FSH binds to FSH receptors located on the cell surface of granulosa cells. It stimulates aromatase activity, increases granulosa cell proliferation, and induces the growth of follicles to the mid-antral stage. FSH is suppressed by estradiol and progesterone (described below).[21,23] In antral follicles >6 mm, aromatase converts androgens produced in theca cells to estrogens (e.g. estradiol).[31,32]

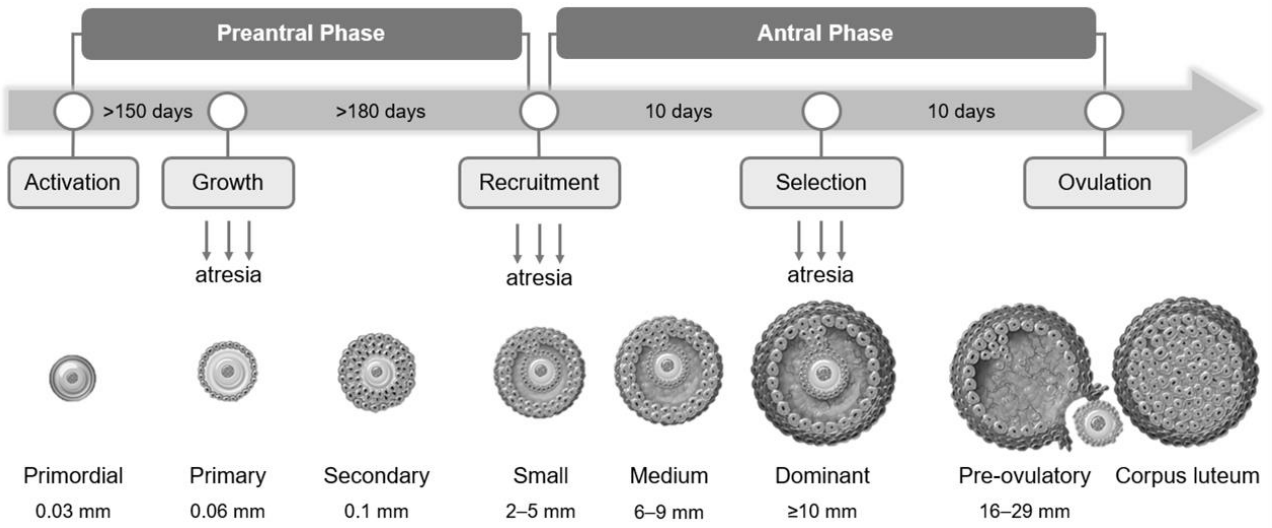
Estradiol is a steroid hormone synthesized from cholesterol and produced by the granulosa cells of antral follicles. Estradiol at low concentrations provides negative feedback to the anterior pituitary, thereby suppressing gonadotropin secretion and continued follicle growth. Estradiol at high concentrations may provide positive feedback to the pituitary, but the mechanisms of this action are still uncertain.[29] Progesterone is a steroid hormone synthesized in the luteinized granulosa and theca cells of the pre-ovulatory follicle and CL. It is mainly under the control of LH and provides negative feedback to the anterior pituitary, thereby suppressing gonadotropin secretion during the luteal phase.[30]

Ovarian Follicular Development

Folliculogenesis is a very long process in women.[33] It begins as early as the fourth month of embryonic development, when somatic cells surround the oocytes and form primordial follicles.[27,34] Follicles at this stage are arrested in meiosis and comprise the ovarian reserve, which provides a woman with reproductive potential for her lifetime. Depletion of the ovarian reserve begins during gestation and continues through menopause.[34] Prior to puberty, primordial follicles progress from the resting (0.03 mm) to growing state (0.06 mm) (Figure P.1). This process takes approximately five months; some follicles initiate growth immediately, while others remain quiescent for years.[35–37] Pre-antral follicles (0.06–0.2 mm) grow independent of gonadotropin

support. The process takes approximately six months.[33,27] Follicles then form a fluid-filled cavity (i.e. antrum) at a diameter of 0.2–0.4 mm and become responsive to gonadotropins. Follicles undergo atresia (or regression) in the absence of gonadotropins.[27,35] Maturation of the hypothalamic-pituitary-ovarian axis at puberty enables the cyclic development of antral follicles to 2 mm or larger[38] (Figure P.1). The presence of an antrum allows this process to be visualized on ultrasonography.[22,39] Descriptions of the stages of folliculogenesis are provided in more detail below.

Figure P.1. Normal ovarian folliculogenesis in women. *Distinct phases, key events, and overall timing of folliculogenesis are depicted. Follicular graphics were obtained from Ansh Labs.*



Pre-Antral Phase

Primordial (or resting) follicles are 0.03 mm in diameter and characterized by a single layer of flattened granulosa cells.[33] Activation of follicular growth to the primary stage (0.06 mm) involves the proliferation and differentiation of the granulosa cells, as well as the actions of multiple hormones and growth factors.[22,27,40] Follicular growth is driven by further replication of the granulosa cells and an acquisition of theca cells.[21] At the secondary stage (0.1 mm), the follicular vasculature develops, and the granulosa and theca cells begin to express receptors for

gonadotropins and ovarian steroid hormones.[27,33,35] Pre-antral follicles are gonadotropin-sensitive, but their development is largely mediated by paracrine and autocrine factors secreted from the oocyte and surrounding cells.[37]

Antral Phase

At the early antral stage (0.2–2 mm), an antrum forms from fluid collections within the follicle.[41] Antral follicles then progress towards ovulation through three physiologic classes: (1) small (2–5 mm), (2) medium (6–9 mm), and (3) large (≥10 mm). Such growth is dynamic, and reflects continued replication of the granulosa cells, wherein each class is distinguished by the number of granulosa cells present.[33]

A cohort of small antral follicles (2–5 mm) is “recruited” from the pre-antral pool at least once during the menstrual cycle.[35,42] The process of recruitment typically occurs once during the late-luteal or early follicular phase.[35] These follicles are dependent on gonadotropins for continued growth and will undergo atresia in the absence of sufficient stimulation.[21] Atresia occurs via apoptosis, which is a form of programmed cell death. During the reproductive years, >99% of growing follicles will undergo atresia, whereas only 1% will ovulate.[34] An increase in circulating concentrations of FSH above a critical threshold is required to stimulate antral follicle growth to the mid-antral (6–9 mm) stage.[43]

Medium antral follicles grow synchronously with one another until a single dominant follicle (≥10 mm) is “selected” for preferential growth. Multiple cohorts of medium antral follicles may emerge and regress throughout the menstrual cycle, but the process of selection occurs once in the early- to mid-follicular phase, thereby leading to ovulation.[43,44] The dominant and largest ‘subordinate’ follicles undergo a common growth phase in women. At the time of selection, the dominant follicle begins to ‘diverge’ as it continues to grow, while the remaining follicles undergo atresia.[42–44] Dominant follicle selection is associated with a decrease in circulating concentrations of FSH and an increase in circulating concentrations of estradiol.[29]

Estradiol synthesis by the selected follicle is preceded by LH-stimulated androgen production in theca cells. Aromatization of androgens to estradiol within the growing follicle increases local and systemic estradiol concentrations.[26] Estradiol produced by the dominant follicle stimulates proliferation of the endometrial lining in preparation for ovulation and subsequent conception.[27] Estradiol production also increases LH pulse frequency, which drives aromatase activity. Increased aromatase activity induces LH receptors on granulosa cells and causes the follicle to switch from FSH to LH-dependent growth.[29] Notably, inappropriate LH production or stimulation can inhibit further follicular growth and disrupt oocyte maturation.[32]

The dominant follicle grows at an accelerated rate after selection and attains a pre-ovulatory diameter of 16–29 mm in the late follicular phase.[43–45] Granulosa and theca cells continue to proliferate and antral volume increases.[27] The persistently elevated serum estradiol concentration triggers an LH surge from the anterior pituitary. The LH surge induces final maturation and ovulation of the oocyte from the dominant follicle.[21,29]

A sharp decline in estradiol and increase in progesterone production occurs as enzymes and proteases work to degrade the follicular wall. The granulosa and thecal cells rearrange into the CL.[30] Luteal development involves significant growth and differentiation. Luteinized theca and granulosa cells gain the ability to produce progesterone and retain their abilities to synthesize androgens and estradiol.[30] Angiogenesis represents a significant portion of luteal growth, as the primary function of the CL is to synthetize and secrete progesterone, which nurtures the endometrial lining to provide a conducive environment for implantation.[29,30]

If these events do not occur, the CL undergoes luteolysis (or involution) via apoptosis.[29,30] The corresponding decrease in progesterone and estradiol causes atrophy of the endometrial blood supply and the endometrium begins to degrade.[30] This process is referred to as menstruation. The decrease in progesterone and estradiol also result in an increase in FSH production from the anterior pituitary, which signal the start of the next cycle.[23]

Current Model of Impaired Folliculogenesis in PCOS

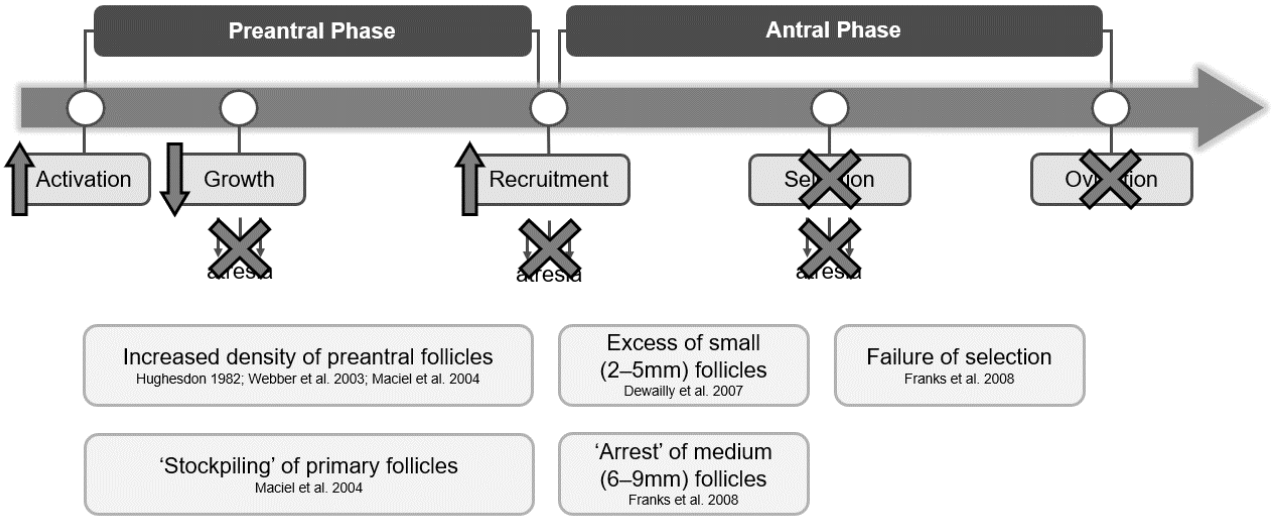
Defects have been identified across the hypothalamic-pituitary-ovarian axis in PCOS.[46,47] Pulsatile secretion of gonadotropin-releasing hormone from the hypothalamus is persistent and rapid in this condition.[25] This pattern favors the synthesis of LH by the anterior pituitary and contributes to an increased LH: FSH ratio.[48] High levels of LH[49] and increased activity of steroidogenic enzymes[50] augment ovarian androgen production in polycystic ovaries, whereas low levels of FSH likely inhibit follicular growth.[46,47] Serial endocrine dynamics have never been described in PCOS. However, patients often demonstrate abnormal endocrine profiles, including elevated circulating concentrations of LH and androgens, and reduced serum concentrations of FSH.[51,52]

Polycystic ovarian morphology reflects aspects of disordered folliculogenesis in PCOS.[47] The morphological hallmark is an increased number of growing follicles, from the primary stage (0.06 mm) to the antral stage (2–5 mm) (Figure P.2).[53–55] Histologic studies have confirmed that polycystic ovaries contain 2–3 times more primary, secondary, and small antral follicles than normal ovaries.[53–55] Specifically, primary follicles are “stockpiled” in this condition.[54,55] Pre-antral “follicular excess” may arise from increased activation of follicular growth from the primordial pool,[54] slower maturation of primary follicles,[55] and/or decreased atresia.[56] Simultaneously, ultrasonographic studies have revealed a paucity of medium- and large antral follicles in polycystic ovaries.[57] Larger follicles are presumably arrested in development due to impaired theca and granulosa cell function (Figure P.2).[46,47]

Namely, theca cells from polycystic ovaries have shown an enhanced capacity to produce androgens in response to luteinizing hormone (LH).[49] Androgens may then promote theca and granulosa cell proliferation and stimulate excessive growth to the selectable stage (2–5 mm).[47] Further, granulosa cells from polycystic ovaries have shown premature responsiveness to LH in follicles as small as 4 mm. This implies the early acquisition of LH receptors, which in normal ovaries, does not occur until the time of selection (i.e. at ~10 mm).[22,58] The theory is that these

functional abnormalities collectively result in an inhibition of terminal follicular growth at the 6–9 mm stage.[47,46] Androgens may further inhibit atresia after follicles stop growing and therefore contribute to a state of follicular "persistence."[59,60] Together, these abnormalities are thought to inhibit selection and ovulation (Figure P.2).[46,47]

Figure P.2. Current model of impaired folliculogenesis in PCOS. *Proposed abnormalities in distinct phases and key events of folliculogenesis are depicted. Up and down arrows refer to increased and decreased activity, respectively, and "X's" refer to absent events. Follicular graphics were obtained from Ansh Labs.*



The corollary to follicular excess, arrest, and persistence is the concept that selection and ovulation cannot occur in women with PCOS.[47,46] However, these phenomena do not provide a unanimous explanation for the disordered antral folliculogenesis in this condition, because some follicles can periodically ovulate during natural cycles or be "rescued" through pharmacologic intervention.[47,46]

Rationale for the Current Studies

Longitudinal studies are ultimately needed to corroborate the degree to which antral follicle development is impaired in PCOS. To that end, a prospective cohort study was conducted to

characterize antral folliculogenesis in women with anovulatory phenotypes of PCOS. Owing to our observation of anovulatory and sporadic ovulatory cycles, **Part 1** of this dissertation aimed: (1) To determine the extent to which follicular excess, arrest, and persistence are consistent findings in PCOS ***(Chapter 1)***; and (2) To evaluate the growth kinetics of ovulatory follicles, and (3) To identify the clinical factors that are associated with sporadic ovulation in PCOS ***(Chapter 2)***.

Part 2 of this dissertation built on our new understanding of antral folliculogenesis and ovulatory potential in PCOS (**Part 1**) to guide diagnostic and treatment strategies better in this condition. Follicular excess is assumed to be a constant feature over time, and metrics that reflect follicle number are recommended to define polycystic ovarian morphology on ultrasound.[19] However, it is unclear whether follicle development during anovulatory or sporadic ovulatory cycles can confound the potential of sonographic markers to detect PCOS consistently. Thus, **Part 2** of this dissertation aimed to evaluate any impact of a dominant follicle or a corpus luteum on the morphologic diagnosis of PCOS ***(Chapter 3).*** Moreover, because most patients are overweight or obese,[61] modest weight loss (5–10%) is recommended to improve the likelihood of ovulation, both naturally and in response to assisted reproduction therapies.[62] Data on the significance of sporadic ovulation are limited ***(Chapter 2),*** but are necessary to understand the extent to which current first-line strategies can actually impact ovulatory function in women with PCOS. As such, **Part 2** of this dissertation further aimed: (1) To assess the evidence surrounding the effectiveness of hypocaloric dietary intervention to normalize ovulatory cyclicity, and (2) To provide recommendations to strengthen research in this area ***(Chapter 4).***

CHAPTER 1

A NEW MODEL OF ANTRAL FOLLICULOGENESIS IN ANOVULATORY WOMEN WITH POLYCYSTIC OVARY SYNDROME

ABSTRACT

Objective. On ultrasonography, polycystic ovarian morphology is characterized by an abundance of small and medium-sized antral follicles. It is posited that antral follicles accumulate in the polycystic ovary as the result of impaired follicular maturation and decreased atresia. These phenomena have long been implicated in the mechanism of anovulation in polycystic ovary syndrome (PCOS), but have never been explored in vivo. The objectives of this study were to evaluate antral follicle development in women with PCOS and determine the degree to which follicular excess, arrest, and/or persistence are consistent findings in the condition.

Methods. Women with PCOS (n=11) and regular ovulatory cycles (n=11) were prospectively evaluated by serial transvaginal ultrasonography. Antral follicle number and diameter (≥2 mm) were quantified every other day for 4–5 weeks. Follicle size populations and growth kinetics were compared between groups.

Results. Antral follicle counts were substantially greater in women with PCOS compared to Controls on any given day (105 vs. 49 follicles, P<0.01). Differences between groups were driven by a higher number of both 2–5 mm (P<0.01) and 6–9 mm follicles (P=0.02) in women with PCOS. Antral follicle development in PCOS appeared disorganized, with numerous follicles randomly emerging and regressing throughout the scanning interval. Uniquely identified follicles grew to smaller maximal diameters in women with PCOS compared to Controls (10 vs. 23 mm, P<0.01).

This reflected shorter growth phases (P<0.01), but similar static (P=0.87) and regression phases (P=0.07). Overall, growth and regression rates did not differ between groups.

Conclusions. Antral follicle growth and regression can be detected on ultrasonography in women with PCOS, despite the failure of dominant follicle selection and ovulation. Documentation of more frequent turnover of follicles in polycystic versus normal ovaries challenges the traditional theory of follicular persistence in PCOS.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the main cause of ovulatory dysfunction in women, but the mechanism of anovulation is unknown. Most insight into disordered folliculogenesis in PCOS has been garnered from histologic studies of pre- and early antral follicles. Authors have described an “excess” of small follicles,[53,55,54] and the premature “arrest”[58,63] and “persistence” of larger follicles.[59,60] However, very few studies have focused on advanced stages of follicular development (i.e. ≥2 mm); actual disruptions in selection and dominance have remained largely unexplored. Such investigations are critical to inform appropriately the treatment strategies for anovulation in PCOS.

Polycystic ovarian morphology reflects aspects of disordered folliculogenesis in PCOS.[47] The morphological hallmark is an increased number of growing follicles, from the primary stage (0.06 mm) to the antral stage (2–5 mm). Histologic studies have confirmed that primary follicles are “stockpiled” in polycystic ovaries.[55,54] Moreover, the total population of primary, secondary, and antral follicles is 2–3-fold greater in polycystic versus normal ovaries.[53,55,54] This follicular excess is thought to reflect increased initiation of follicle growth from the primordial pool,[54] slower maturation of primary follicles,[55] and/or decreased follicle loss by atresia.[56]

Despite the increased size of the growing pool, there is an apparent failure of normal selection and ovulation.[47,46] Polycystic ovarian morphology is simultaneously characterized by a paucity of medium- and large follicles (>5 mm).[57] Any large follicles present in the ovaries are presumably “arrested” in development due to impaired theca and granulosa cell function.[47,46] Namely, theca cells from polycystic ovaries have shown an enhanced capacity to produce androgens in response to luteinizing hormone (LH).[49] Androgens may then promote theca and granulosa cell proliferation and stimulate excessive growth to the selectable stage (2–5 mm).[47] Further, granulosa cells from polycystic ovaries have shown premature responsiveness to LH in follicles as small as 4 mm. This implies the early acquisition of LH receptors, which in normal ovaries, does not occur until the time of selection (i.e. at ~10 mm).[22,58] The theory is that these

functional abnormalities collectively result in an inhibition of terminal follicular growth at the 6–9 mm stage.[47,46] Androgens may further inhibit atresia after follicles stop growing and therefore contribute to a state of follicular "persistence."[59,60]

There is still a great deal to learn about disordered folliculogenesis in PCOS. First, it is not currently known whether antral follicle populations change over time or whether the follicular excess represents a constant phenomenon in PCOS. Second, it is not known whether the paucity of larger follicles in polycystic ovaries reflects follicle arrest or some other defect. Follicle arrest has never been observed in vivo, which is notable, because sporadic ovulation can occasionally occur and follicle growth can be stimulated by pharmacologic therapy.[47,46] Third, it is not known whether follicles are protected from atresia in polycystic ovaries and whether they can "persist" at their maximum diameters for an extended period of time.[59,60] Elucidating the defects that exist in PCOS is likely to improve treatment strategies for patients. This is particularly important in the context of assisted reproduction technologies, wherein women with PCOS have increased risk for ovarian hyperstimulation syndrome (OHSS) and often have resistance to first-line ovulation induction protocols (i.e. clomiphene citrate).[5]

High-resolution transvaginal ultrasonography provides a non-invasive means to answer these questions, as it allows for the reliable detection of antral follicles ≥2 mm.[39] Follicular growth, atresia, and ovulation have been serially evaluated in women with regular menstrual cycles.[44,43,45] and the established techniques can be readily applied to PCOS. Recent observations in our laboratory revealed substantial changes in follicle size populations on ultrasound scans performed one month apart in women with PCOS.[64] These findings led to the notion that polycystic ovarian morphology may not solely represent a condition of follicular excess, arrest, and persistence. To that end, the objective of this study was to characterize follicle growth and turnover during an anovulatory interval in women with PCOS. We hypothesized that an active component of antral folliculogenesis would be captured on ultrasonography amidst follicular excess in this condition.

Based on our preliminary observations,[64] we further hypothesized that frequent changes in the number and diameter of follicles would reflect follicular arrest, but not persistence.

METHODS

Ethical Considerations

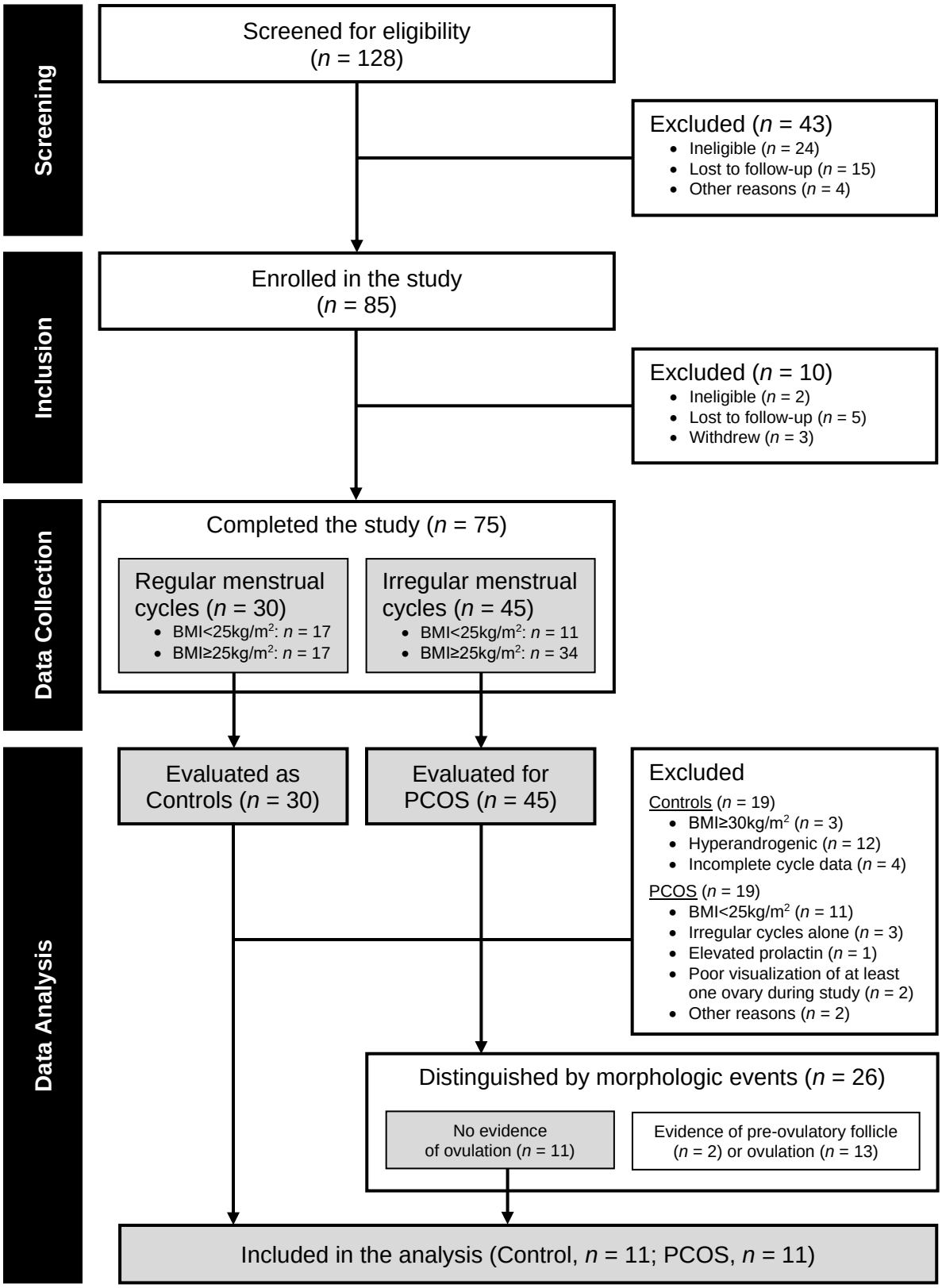
Data from two separate, prospective, ongoing trials were evaluated in this study. Both research protocols were individually approved by the Institutional Review Board at Cornell University and the trials were registered at ClinicalTrials.gov (NCT01927432, NCT01785719). Informed consent was obtained from all participants before the study procedures were initiated.

Study Participants

One hundred twenty-eight women were recruited from the general population between 2009 and 2016 (Figure 1.1). Paper and electronic advertisements were circulated throughout Cornell University and the surrounding Southern Tier Counties of New York State. Recruitment efforts were targeted to establish two distinct cohorts: (1) women with regular menstrual cycles (every 21–35 days) and (2) women with irregular menstrual cycles (>35 days apart). A major goal was to enroll an equal number of normal weight (body mass index, BMI, 18.5–24.9 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2), and obese (BMI ≥30.0 kg/m^2) women in each group. Potential participants were screened using general health and reproductive history, as well as physical and endocrine examination. Women of reproductive age (18–38 years), with consistent and optimal visualization of both ovaries on ultrasonography, were eligible to participate. Exclusion criteria were use of medications known or suspected to interfere with reproductive function in the two months prior to the study (e.g. hormonal contraception, fertility medications, antibiotics, antivirals); pregnancy or lactation in the six months prior to the study; history of premature ovarian failure or surgery; and preexisting medical conditions expected to interfere with study participation or outcomes (e.g. endometriosis, vaginal abnormalities, bleeding disorders, pre-diabetes, diabetes mellitus). Women with untreated thyroid abnormalities or hyperprolactinemia were excluded through appropriate hormone assessment, because these conditions mimic features of PCOS.[7]

Women who completed the study were retrospectively evaluated for inclusion in the present analysis (Figure 1.1). Cohorts of interest included: (1) overweight and obese women with anovulatory phenotypes of PCOS and (2) healthy controls. Anovulatory phenotypes of PCOS were identified based on the 2003 Rotterdam criteria.[8,9] Oligo- or anovulation was judged by a self-reported history of irregular menstrual cycles (i.e. >35 days apart) and confirmed with ultrasound monitoring of ovarian follicular development during the study. To achieve the objectives of the present analysis, women with a pre-ovulatory follicle (>14 mm) or sporadic ovulation during the study were excluded (Figure 1.1). Hyperandrogenism was defined as a modified hirsutism score ≥7 or total testosterone concentration ≥65.4 ng/dL. These markers of androgen status were measured as described previously by our group,[65] and internal thresholds were derived from the 95th percentiles of modified hirsutism scores and total testosterone concentrations in healthy women. Polycystic ovarian morphology was detected on transvaginal ultrasonography by a mean follicle number per ovary ≥25 or mean ovarian volume ≥10 mL.[19,66] Lean women (BMI 18.5–24.9 kg/m^2) with PCOS were excluded from the present analysis, based on evidence that adiposity is involved in the mechanism of anovulation in PCOS[46,67] and that reproductive dysfunction worsens at BMIs ≥25 kg/m^2.[68] Normal- and overweight (BMI <30 kg/m^2) women with regular ovulatory cycles and normal androgen status were designated as Controls. Controls were matched for age, but not BMI, based on evidence that obesity (BMI ≥30 kg/m^2) adversely affects ovulatory function (Figure 1.1.).[69,70]

Figure 1.1. Flow of participants through the observational cohort study (2009–2016) and present analysis. *Shaded boxes designate the cohorts from which participants were selected for the present analysis. Abbreviations: PCOS, polycystic ovary syndrome; BMI, body mass index.*



Ultrasonographic Measurements

Serial transvaginal ultrasonography was used prospectively to evaluate antral follicle development (≥2 mm) in PCOS and Controls. Approaches were largely based on studies performed in healthy women by Baerwald and colleagues,[44,71,72] but were modified where appropriate to evaluate outcomes in women with cycle irregularity. In women with PCOS, scans began at a random time and were performed every other day for 4–5 weeks. In Controls, scans were initiated in the mid-follicular phase and continued every other day for one inter-ovulatory interval (IOI). An IOI was defined as the time between consecutive ovulations and represented the luteal phase of one cycle followed by the follicular phase of the next cycle.[44,72] This scanning regimen was necessary to capture the complete trajectory of follicular development, because ovulatory follicles can begin to grow as early as the luteal phase of the previous cycle.[44,43] Follicular growth was closely monitored in real-time in both groups. If a large follicle (≥14 mm) was detected, then ultrasound examinations were performed daily until its regression or ovulation. Ovulation was identified by subsequent observation of a corpus luteum.[73,74]

Scans were performed by one of four operators using a GE Voluson E8 Expert System and 6–12 MHz 3D/4D transducer (GE Healthcare, Milwaukee, WI). Whole ovaries were imaged from their inner to outer margins in the longitudinal plane. Three-dimensional datasets were acquired using customized settings and the automated volume modality. Two-dimensional cineloops of each ovary in its sagittal and transverse planes were then extracted (Slice Thickness: 0.5 mm) and archived for offline image analysis. Cineloops were evaluated by a single investigator with customized imaging software (Sante DICOM Editor, Santesoft LTD, Athens, Greece). Antral follicle number and diameter were assessed for each ovary and visit of the scanning interval. Reliable follicle counts were achieved with the grid system approach, as previously described by our group.[75] Diameter measurements were obtained in the largest cross-sectional view of the follicle and calculated as the average of its two orthogonal dimensions (i.e. length × width). If a large follicle (≥10 mm) was detected, then the same measurements were repeated in a second

plane and the four dimensions were averaged. Mean follicle diameter was rounded to the nearest whole number.[72]

Growth and regression profiles of individual follicles were assessed using the Identity Method.[44,71,72] Briefly, the diameter and location of follicles that grew to ≥4 mm were sketched on paper for each ovary and visit of the scanning interval. Locations of individual follicles were designated by anatomical landmarks and positions relative to other follicles within the ovary and cineloop. Each follicle that grew to ≥7 mm was alphabetized (i.e. uniquely identified), and any changes in its diameter were tracked over time from day of first detection (at 4–5 mm) to day of last detection (at 4–5 mm).[44,71,72]

Growth and regression rates of individual follicles were then determined. Sonographic presence was defined as the time between the first and last day of detection of a follicle.[76] The growth phase of a follicle began on the day of first detection and ended on the day of maximal diameter.[45] The regression phase of a follicle began on the day of maximal diameter and ended on the day of last detection.[45,76] Static phases were identified when a follicle was detected within 1 mm of its maximal diameter for at least three days (i.e. two visits).[45,76] Data from the first and last day of a static phase coincided with the end of the growth phase and beginning of the regression phase, respectively, and were included in calculations of growth and regression rates.[76]

Follicle number and diameter data were combined for both ovaries.[44,72] The total number and proportion of follicles detected in different diameter categories were graphed for each woman over the scanning interval. Follicle populations of physiologic interest included: ≥2 mm, 2–9 mm, 2–5 mm, 6–9 mm, and ≥10 mm. Diameter profiles of uniquely identified follicles were also graphed for each woman.

Biochemical Measurements

Fasting blood samples were drawn on a single day of the study. Measurements were performed in the early follicular phase in Controls (i.e. days 1–8 of the menstrual cycle) or at a random time in women with PCOS. Time points were standardized in both groups such that no dominant follicles or active corpora lutea were present. Blood was collected into a clot-activated tube and allowed to sit at room temperature for 30–60 minutes. Serum was then isolated by centrifugation and stored at –80°C until analysis. Samples were shipped to an affiliate of the Centers for Disease Control and Prevention Hormone Standardization Program, and total testosterone was measured by liquid chromatography tandem mass spectrometry (Brigham Research Assay Core, Boston, MA). The inter-assay coefficient of variation was 6.4% and the measurement range was 1.00–2,000 ng/dl.[77] Sex hormone binding globulin was measured in an aliquot of the same sample by chemiluminescence immunoassay (Immulite 2000; Siemens Medical Solutions Diagnostics, Deerfield, IL) at an internal clinical chemistry lab (Cornell University, Ithaca, NY). Intra- and inter-assay coefficients of variation were 3.1% and 5.1%, respectively. Free androgen index was calculated as: testosterone (nmol/l) / SHBG (nmol/l) × 100.

Statistical Analysis

Statistical analyses were performed with JMP Pro 12.0.1. (SAS Institute, Cary, NC, USA). The threshold for statistical significance was set at $P \leq 0.05$. Normality was determined visually with histograms or residual plots. Skewed data were evaluated after logarithmic transformation where appropriate.

Demographic and diagnostic features were compared between groups using two-sample t-tests. Longitudinal profiles of follicle number and diameter were evaluated with combined and group-specific mixed models. Data were centralized to the day of the first study visit (PCOS) or ovulation (Control) and normalized over the mean scanning interval. Participant number was included as a random effect in each of the following models. Combined models were used to

assess between-group differences in the mean number of ≥2 mm, 2–9 mm, 2–5 mm, and 6–9 mm follicles. In these models, PCOS diagnosis was designated as a categorical fixed effect. A “group” effect was interpreted as evidence of follicular excess in women with PCOS compared to Controls. Group-specific models were used to assess within-group changes in the mean number of ≥2 mm, 2–5 mm, and 6–9 mm follicles. In these models, time was designated as a continuous fixed effect (i.e. day of the scanning interval) and fit to a linear, quadratic, cubic, or quartic function where appropriate. Participant number, crossed with time, was added as a random effect. A “day” effect in the number of 2–5 mm or 6–9 mm follicles was interpreted as evidence of follicular recruitment during the scanning interval. In the event of a day effect in either follicle size population, additional models were conducted to clarify when recruitment had occurred. Time was designated as a categorical fixed effect (i.e. cycle phase). Anovulatory intervals were binned into three sequential 9–10-day intervals. IOIs were divided into the early follicular phase (with all follicles <10 mm), mid- to late-follicular phase (with evidence of at least one dominant or pre-ovulatory follicle), and luteal phase (with evidence of a corpus luteum). A “phase” effect in the number of 2–5 mm or 6–9 mm follicles was then interpreted as evidence that recruitment had occurred during a specific phase. Mean follicle populations were compared between phases with post-hoc Tukey’s honest significant difference tests. The restricted maximum likelihood approach was used to identify the degree of intra-cycle versus inter-individual variation in each model. Last, combined models were used to assess between-group differences in the mean growth and regression profiles of uniquely identified follicles. As in the other combined models, PCOS diagnosis was designated as a categorical fixed effect. Follicle number, nested within participant number, was added as a random effect. A “group” effect in any growth-related endpoint was used to evaluate follicle arrest and turnover.

RESULTS

Characteristics of Study Participants

Study enrollment is described in Figure 1.1. Seventy-five women completed the study procedures. Twenty-two women represented the cohorts of interest and were included in the present analysis (*n*=11 women with PCOS, *n*=11 Controls) (Figure 1.1). General demographic and diagnostic features are compared between groups in Table 1.1. Women with PCOS differed from Controls across all features (*P*<0.05) except age (*P*=0.18) (Table 1.1).

Table 1.1. Characteristics of study participants

	PCOS (*n* = 11)	**Control (*n* = 11)**
Age (y)	26 ± 4 (21, 34)[a]	29 ± 6 (19, 38)[a]
Body mass index (kg/m^2)	36.3 ± 8.0 (26.4, 48.4)[a]	23.9 ± 1.9 (21.2, 27.3)[b]
Menstrual cycle length (d)	197.3 ± 138.4 (50, 365)[a‡]	29 ± 2 (24, 31)[b]
Hirsutism score	8 ± 4 (1, 15)[a]	2 ± 3 (0, 6)[b]
Total testosterone (ng/dL)	67.8 ± 27.9 (32.9, 118.0)[a]	30.5 ± 9.6 (17.6, 43.6)[b]
Free androgen index	11 ± 6 (4, 23)[a]	2 ± 1 (1, 3)[b]
Mean follicle number per ovary	50 ± 22 (14, 80)[a]	25 ± 9 (11, 35)[b]
Mean ovarian volume (mL)	16 ± 4 (7, 21)[a]	6 ± 4 (2, 12)[b]

Data are presented as mean ± SD (minimum, maximum). [a,b] Within rows, uncommon superscripts denote significant differences between groups, p<0.05. Diagnostic endpoints were evaluated on a single day of the scanning interval and with respect to stage of cycle. [‡] Data related to menstrual cycle length were censored to the year prior to enrollment (i.e. 365 days).

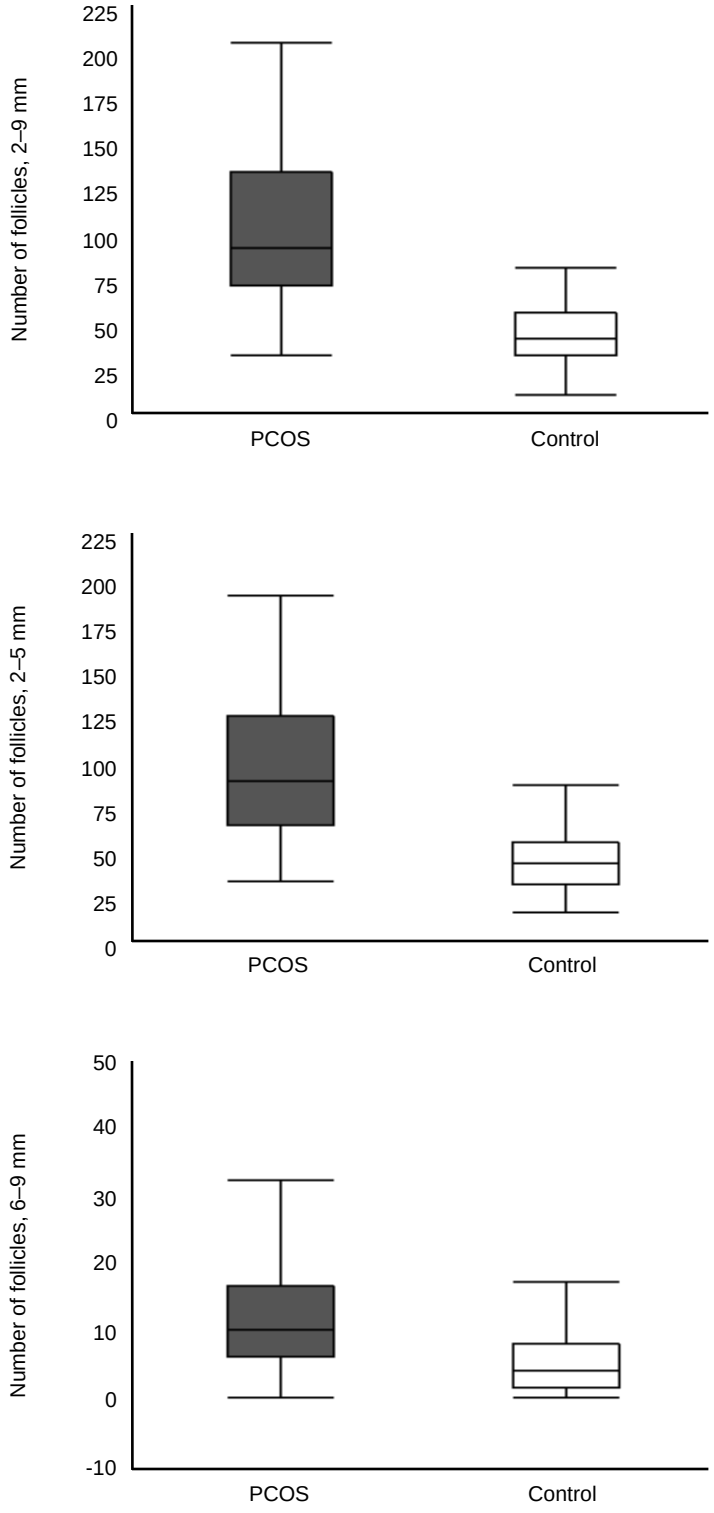
Women with PCOS were scanned for an average of 30 ± 3 days. Four women with PCOS reported amenorrhea and an absence of natural menstruation for at least one year prior to enrollment (Table 1.1: data were censored at 365 days). The remaining seven women reported oligomenorrhea with last menses having occurred between four and 60 days before the first study visit (mean ± SD: 25 ± 21 days). None of the women with PCOS showed ultrasonographic

evidence of recent ovulation during the scanning interval. Controls demonstrated normal IOI (mean ± SD: 28 ± 4 days), follicular-phase (mean ± SD: 17 ± 4 days), and luteal-phase lengths (mean ± SEM: 13 ± 1 days).[44,78] Selection and ovulation of a dominant follicle was detected at least twice in each woman (i.e. at the beginning and end of the IOI).

Evaluation of Follicular Excess

Distributions of follicle populations for the entire scanning interval are presented in Figure 1.2. In general, antral follicle counts ranged from 37 to 209 follicles in women with PCOS and from 15 to 85 follicles in Controls (Figure 1.2). Group-specific mixed models identified higher mean estimates for follicle number over time in women with PCOS ($\beta_{Intercept}$: 105; SE: 12; 95% CI: 78–133) compared to Controls ($\beta_{Intercept}$: 49; SE: 4; 95% CI: 40–59). Combined analyses also revealed consistent evidence of follicular excess in PCOS. On any given day, more follicles (2–9 mm) were detected in women with PCOS compared to Controls (β_{PCOS}: 28; SE: 7; P_{PCOS}<0.01; 95% CI: 15–42). These differences were attributed to a greater number of both small (2–5 mm) (β_{PCOS}: 25 (SE: 6); P_{PCOS}<0.01; 95% CI: 13–38) and medium follicles (6–9 mm) (β_{PCOS}: 3; SE: 1; P_{PCOS}=0.02; 95% CI: 1–5) in PCOS.

Figure 1.2. Distributions of follicle number for the entire scanning interval in women with PCOS and controls. *Box-and-whisker plots for the 2–9 mm, 2–5 mm, and 6–9 mm diameter categories are shown. The box represents the 25th and 75th percentiles and the horizontal line within the box represents the median. The 5th to 95th percentile range is reflected by the vertical bars. Outliers, if any, are denoted by dots.*



Evaluation of Follicle Recruitment

Mean profiles of follicle size populations are illustrated in Figure 1.3 and 1.4. Visually, antral follicle counts appeared constant over time in women with PCOS (Figure 1.3, A) and Controls (Figure 1.3, B). Group-specific models confirmed these observations. There was no effect of day on the number of follicles detected in either group (PCOS: P_{Day}=0.23; Controls: P_{Day}=0.98).

Follicle diameter appeared constant over time in PCOS (Figure 1.4, A). In line with these observations, there was no effect of day on the number of small (P_{Day}=0.20) or medium follicles (P_{Day}=0.17) detected in this group. There was a small increase in the proportion of small follicles throughout the scanning interval (β_{Day}: 0.25; SE: 0.10; P_{Day}=0.01; 95% CI: 0.05–0.44), but there were no changes in the proportion of medium follicles (P_{Day}=0.11). In PCOS, most of the variation in follicle populations was attributed to inter-individual differences (2–5 mm: 92%; 6–9mm: 75%), rather than intra-cycle fluctuation (2–5 mm: 8%; 6–9 mm: 25%). Collectively, these findings were not consistent with evidence of normal or excessive recruitment in women with PCOS.

By contrast, follicle diameter appeared to fluctuate over time in Controls (Figure 1.4, B). Similar to previous reports,[44,43] changes in diameter were non-random and dynamic. There was an effect of day on the number of both small (P_{Day}<0.01) and medium follicles (P_{Day}<0.01) in this group. The number of small follicles showed a significant peak in the luteal phase (LS Mean ± SE: 47 ± 4 follicles) and decreased by an average of 6 to 8 follicles during the early (P_{Phase}<0.01) and mid-to-late follicular phases (P_{Phase}<0.01). Concurrently, the number of medium follicles showed a significant nadir in the luteal phase (LS Mean ± SE: 4 ± 1 follicles) and increased by an average of 3 to 4 follicles during the early (P_{Phase}<0.01) and mid-to-late follicular phases (P_{Phase}<0.01) (Figure 1.4, B). Similar outcomes were observed in the proportions of small (P_{Day}<0.01) and medium follicles (P_{Day}<0.01). Unlike women with PCOS, variation in follicle populations was attributed to both intra- and inter-individual differences. Most of the variance in the models was attributed to both intra-cycle fluctuation (2–5 mm: 23%; 6–9 mm: 38%) and inter-

individual differences (2–5 mm: 77%; 6–9 mm: 62%). Collectively, these findings were consistent with evidence of recruitment in the luteal phase and an active transition of follicles between physiologic cohorts preceding ovulation in Controls.

Figure 1.3. Mean profiles of antral follicle count during an anovulatory interval in women with PCOS (A) and an inter-ovulatory interval in controls (B).

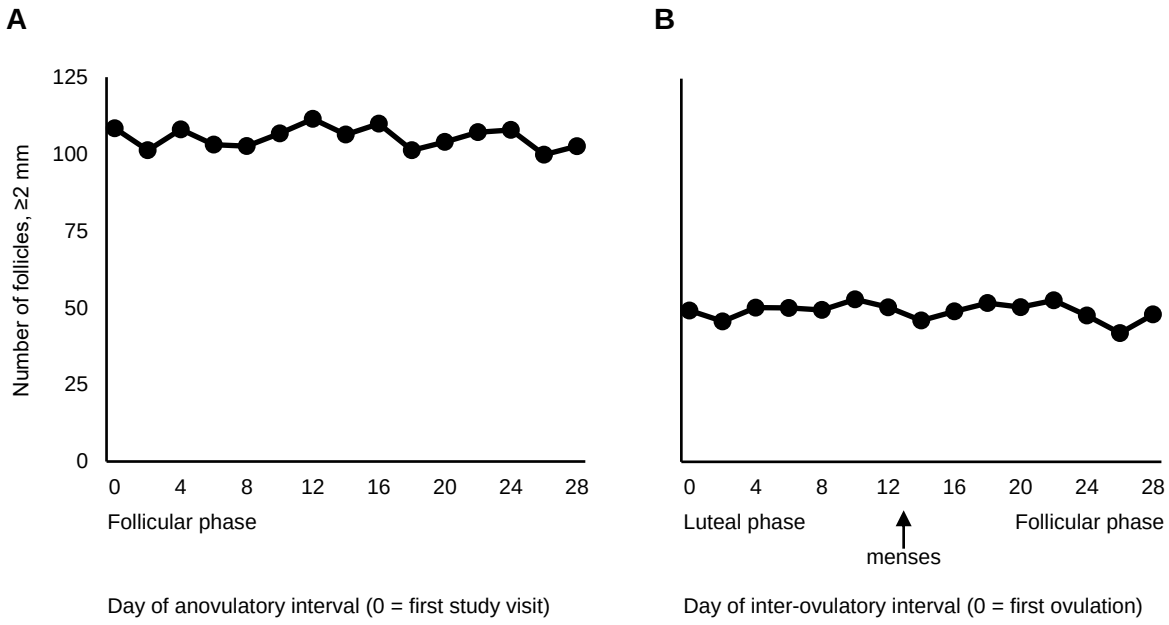
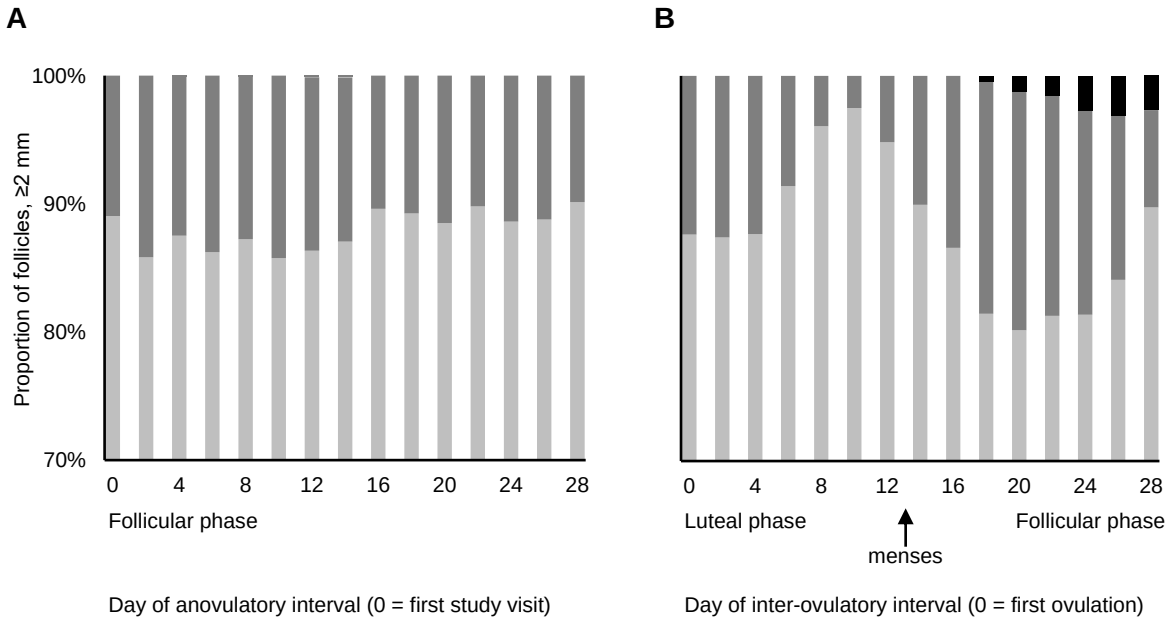


Figure 1.4. Profiles of physiologic cohorts (expressed as mean proportions of the antral follicle count) during an anovulatory interval in women with PCOS (A) and an inter-ovulatory interval in controls (B). *Proportions of 2–5 mm (light gray), 6–9 mm (dark gray), and ≥10 mm (black) follicles are shown. Y-axes are truncated for better visibility of fluctuations over time.*



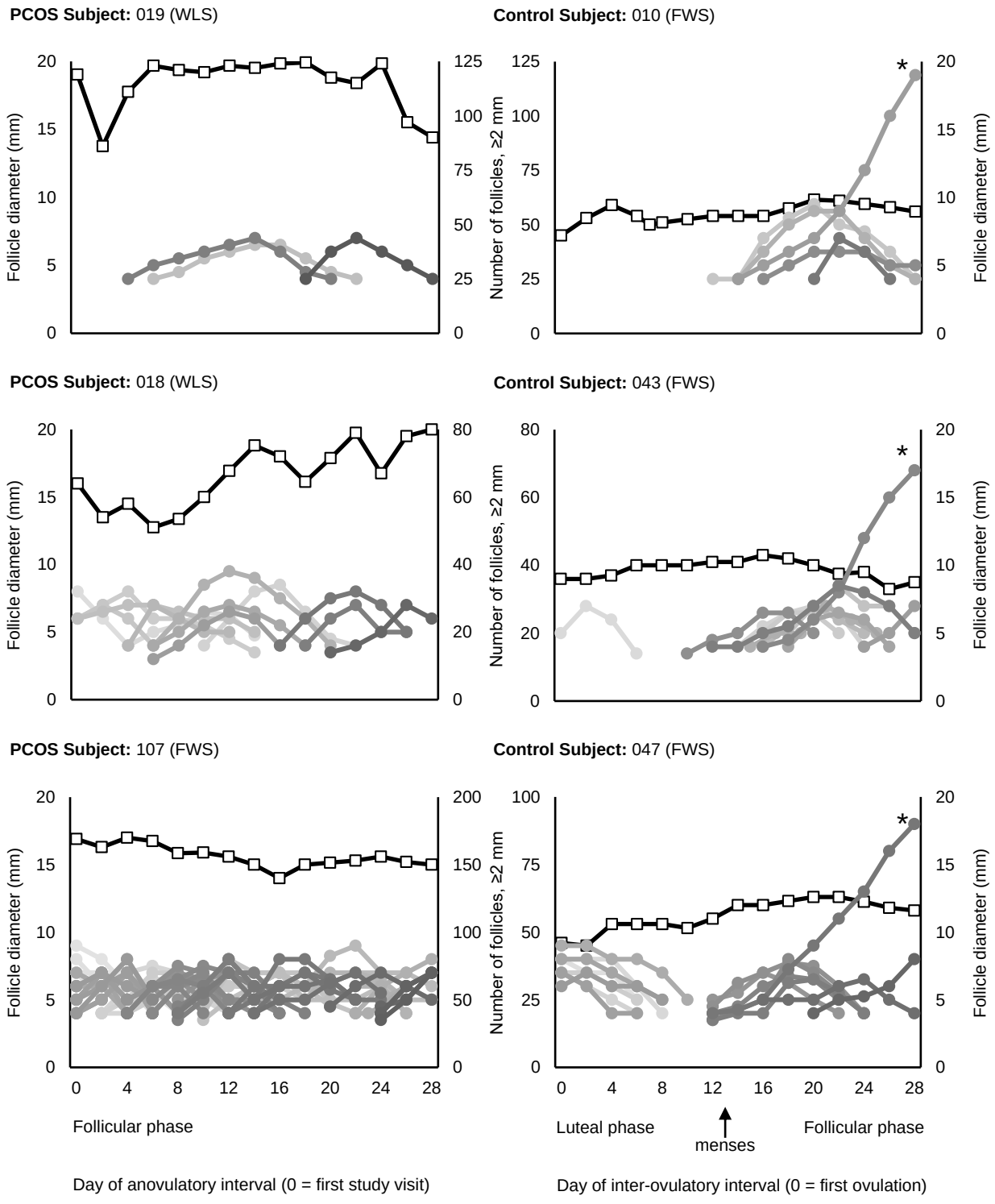
Evaluation of Follicle Arrest and Turnover

Follicles (*n*=501) that grew to ≥7 mm were uniquely identified and tracked over time. Despite significant differences in antral follicle counts between groups (Figure 1.2), there were no differences in the numbers of follicles that were tracked in women with PCOS (mean ± SD: 29 ± 25 follicles) compared to Controls (mean ± SD: 16 ± 11 follicles) (*P*=0.24). On any given day of the scanning interval, the proportion of identified-to-total follicles was also similar between groups (PCOS versus Control, mean ± SD: 31 ± 29% versus 33 ± 17%; *P*=0.46).

Representative plots of antral follicle development are provided for three women with PCOS and three Controls (Figure 1.5). Similar to previous reports,[44,43] follicular growth appeared cyclic and organized in Controls, with distinct cohorts (or groups) of follicles developing at 1–3 points during the IOI (Figure 1.5). Such patterns of growth and regression were not detected in women with PCOS.

Rather, a spectrum of disordered antral follicle development was observed in PCOS (Figure 1.5). At the mild end of the spectrum, fewer than 10% of follicles grew to ≥7 mm. The growth and regression of at least one distinct follicular cohort was detected during the scanning interval (represented by Subject 019). In the middle of the spectrum, between 10 and 30% of follicles grew to ≥7 mm. The growth and regression of at least three follicular cohorts was detected. Yet, unlike the mild group, some growth profiles overlapped with one another and cohorts were more difficult to distinguish (represented by Subject 018). Last, at the severe end of the spectrum, more than 40% of follicles grew to ≥7 mm. Multiple follicles emerged and regressed at different times during the scanning interval. All growth profiles overlapped with one another, and individual cohorts were impossible to resolve (represented by Subject 107). Across the spectrum, however, there was subjective evidence of more frequent follicular turnover in PCOS (Figure 1.5).

Figure 1.5. Representative profiles of follicle growth and antral follicle count during an anovulatory interval in women with PCOS (left) and an inter-ovulatory interval in controls (right). *Each uniquely identified follicle is represented by a different gray line. Note that all follicles that grew to ≥7 mm could be individually tracked over time using the Identity Method. Asterisks indicate ovulatory follicles. The antral follicle count is represented by a black line.*



Complete growth and regression profiles (from first to last day of detection) were available for 177 follicles in women with PCOS and 121 follicles in Controls. Growth and regression parameters are presented in Table 1.2. Follicles grew to smaller maximum diameters in women with PCOS compared to controls (P<0.01). These findings reflected differences in ovulatory status between groups and provided ultrasonographic evidence of follicular arrest in PCOS. Follicles were also detected for a shorter interval in women with PCOS (P<0.01), which reflected shorter growth phases (P<0.01), and similar static (P=0.87) and regression phases (P=0.07). Growth (P=0.11) and regression rates (P=0.85) were similar between groups. These findings were not consistent with the notion of follicular persistence in PCOS (Table 1.2).

Table 1.2. Growth parameters of identified follicles

	PCOS (n = 11)‡	**Control (n = 11)‡**
Sonographic presence (d)	9.9 ± 0.5 (8.8, 10.9)[a]	12.2 ± 0.5 (11.2, 13.3)[b]
Growth phase (d)	3.9 ± 0.2 (3.4, 4.4)[a]	5.7 ± 0.2 (5.2, 6.2)[b]
Growth rate (mm/d)	0.62 ± 0.02 (0.56, 0.67)[a]	0.67 ± 0.02 (0.62, 0.72)[a]
Static phase (d)	3.6 ± 0.4 (2.7, 4.4)[a]	3.7 ± 0.4 (2.8, 4.5)[a]
Maximum diameter (mm)	7.2 ± 0.2 (6.7, 7.7)[a]	8.6 ± 0.2 (8.1, 9.1)[b]
Regression phase (d)	4.2 ± 0.3 (3.6, 4.7)[a]	4.9 ± 0.3 (4.3, 5.5)[a]
Regression rate (mm/d)	–0.57 ± 0.03 (–0.63, –0.52)[a]	–0.57 ± 0.03 (–0.63, –0.51)[a]

Data are presented as LS Means ± SE (95% CI). [a,b] Within rows, uncommon superscripts denote a significant effect of PCOS, p<0.05. ‡ Models included data from 177 follicles in women with PCOS and 121 follicles in Controls.

DISCUSSION

The objective of the present study was to characterize antral follicle growth and turnover in women with PCOS. For the first time, we have documented that follicular excess is constant during an anovulatory interval and cannot be explained by normal or increased recruitment. In addition, we have also revealed that follicles in polycystic ovaries become arrested at the mid-antral stage and undergo more frequent turnover than follicles in healthy ovaries. This new knowledge challenges the traditional theory of follicular persistence in PCOS.

The excess of small, medium, and total antral follicles has long been considered a salient feature in PCOS. This observation is consistent in vitro studies, which have confirmed that polycystic ovaries contain substantially more follicles than normal ovaries.[53,55,54] Our study extends these data to support that follicular excess is also a constant feature over time. The demonstration of non-significant intra-cycle variation in antral follicle counts in PCOS may have important implications for the diagnosis of this condition. Currently, diagnostic evaluations of polycystic ovaries are restricted to the early follicular phase of a natural cycle or pharmacologically-induced withdrawal bleed. This greatly limits the timing to confirm a diagnosis of PCOS and places an additional burden on patients, when menstrual cycle status is uncertain, and medications are needed to immediately control symptomology. Our findings suggest that it may be appropriate to evaluate polycystic ovarian morphology at random times.[19,20] A more direct assessment is needed to confirm this theory. Non-significant intra-cycle variation was also observed in antral follicle counts in our Control cohort. This finding is not consistent with previous evaluations of follicle number during regular menstrual cycles.[79,80] Differences in our study may relate to our use of updated imaging technology[19] and reliable techniques to detect smaller follicles in the ovaries.[75] Yet, we postulate that even significant intra-cycle variation would not confound the potential of the current sonographic definition of polycystic ovarian morphology to detect PCOS.[66]

We did not find evidence of excessive follicular recruitment in women with PCOS. It has been hypothesized that the accumulation of follicles in polycystic ovaries reflects the increased growth of follicles to the small antral stage (2–5 mm). Androgens presumably modulate this process.[47] We, therefore, expected to observe frequent changes in the number of 2–5 mm follicles throughout the anovulatory interval. Although some fluctuation was noted at the individual level, a day effect was not detected for this follicle size population at the group level. The absence of a day effect in the small follicles may have reflected the approach we used to standardize the data over the anovulatory interval. Because it is often difficult to predict menses in women with PCOS, our participants began the study at a random time. Self-reported last menses had occurred anywhere from four to >365 days prior to the first study visit. It is unknown whether follicular development differs in oligo- versus anovulatory women. Such differences may have impacted our ability to detect changes in follicle populations, because “time” was standardized to an arbitrary visit rather than cycle day. We attempted to reconcile this issue by standardizing our data based on the timing of distinct increases in the number of medium-sized follicles in each woman. This approach has been used to align follicular events during regular ovulatory cycles.[44] Yet, surprisingly, when we standardized the data to these time points, we still could not identify changes in follicle populations over time in PCOS (data not shown). The observation that antral follicle development occurred along a spectrum of disorder supports the notion that standardization to distinct time points in antral folliculogenesis may not be possible in PCOS. Future studies may require the identification and use of new methods that can better characterize the spectrum of disordered folliculogenesis in this condition.

We also did not find evidence of normal follicular recruitment in women with PCOS. Controls exhibited an increase in the number of 2–5 mm follicles in the luteal phase, which was consistent with the single recruitment of a follicular cohort.[35] We subsequently observed a transition of follicles between diameter categories, as judged by changes in the numbers and proportions of small and medium follicles. Previous studies have shown that this transition reflects

multiple “waves” of follicular development during an IOI and culminates in the selection and ovulation of a dominant follicle.[44,43] Intra-cycle fluctuation in the small and medium follicles was substantial and significant in Controls (i.e. >20%). But, in women with PCOS, we suspect that the absence of similar fluctuation or transition reflects the known impairments in gonadotropin signaling in this condition.[47,46,25]

Our findings represent the first in vivo evidence of follicular arrest in PCOS. Previously, histologic studies have suggested that follicles in polycystic ovaries can acquire LH receptors at diameters of 8 mm or smaller. This results in premature terminal differentiation of the granulosa cells and halts follicular development beyond the 6–9 mm range.[58] Consistent with these data, we noted that follicles in polycystic ovaries stopped growing at a diameter of 7 mm. Some follicles reached the 9 or 10 mm stage, but this was not a universal finding among women with PCOS. Growth rates of follicles to their maximum diameters did not differ between women with PCOS and Controls. Comparative data are only available for women with regular menstrual cycles.[45] In a recent study performed by Baerwald and colleagues, the mean follicular growth rate during natural cycles was 50% higher than in our Control cohort (1.42 versus 0.67 mm/day).[45] This may reflect our less frequent scanning regimen or revised methods for tracking follicles and defining static phases.[45]

Our findings also represent the first in vivo evidence that follicular persistence does not occur at the antral stages of development in PCOS. We noted that follicles in polycystic ovaries demonstrate similar regression phases and rates to follicles in normal ovaries. This was an unexpected finding, given that decreased atresia has been implicated in the mechanism of follicular excess. The intra-ovarian and systemic factors involved in increased follicular turnover in PCOS are unknown. Importantly, these findings do not exclude the possibility that pre-/small antral follicles (rather than medium follicles) are still protected from atresia by androgens.[60,56] Our data were derived from the Identity Method, which enables an evaluation of larger follicles (>6 mm) in the ovaries. We were able to uniquely identify and track the development of ~30% of

follicles in both the PCOS and Control groups. However, the abundance of antral follicles in polycystic ovaries made it challenging to characterize follicular dynamics in the 2–5 mm range. It is, therefore, possible that we did not capture the complete picture of antral development in PCOS.

We noted a spectrum of disordered follicle development in women with PCOS. This is not surprising given the heterogeneity of the condition itself. Endocrine and metabolic features can vary substantially among patients and exist on a spectrum from mild to severe.[51] Because normal ovarian follicular development is regulated by systemic mechanisms,[22] it is possible that variations in follicle growth reflect variations in biochemical factors among women. This is in line with clinical evidence from pharmacologic interventions for anovulation, which have documented increased risk for OHSS in women with higher antral follicle counts.[81] If follicles can be "rescued" from arrest by administration of exogenous follicle-stimulating hormone,[47] then we hypothesize that OHSS may be more likely in women with a greater proportion of growing follicles (i.e. more severe disorder). Likewise, restored ovulatory cyclicity after dietary intervention[82] may be more likely in women with a smaller proportion of growing follicles (i.e. less severe disorder). These hypotheses merit further investigation.

There were three major limitations to our study. First, cohorts of interest were carefully selected by BMI. We focused on overweight and obese women with PCOS, because adiposity has been implicated in the mechanism of anovulation in this condition.[47,46,67] Most patients are also overweight and consequently experience greater severity of reproductive dysfunction than their normal-weight counterparts.[61,68] However, it is important to acknowledge that adiposity can adversely impact the menstrual cycle in otherwise healthy women.[83,84] Additional studies are needed to determine whether aspects of antral folliculogenesis differ between normal- and overweight women with PCOS. Second, we relied on the broadest diagnostic criteria to define PCOS. While these Rotterdam criteria are recommended in clinical practice and research,[6] they also recognize a controversial normoandrogenic anovulatory phenotype.[14,15] Women with "Mild PCOS" experience less severe reproductive disturbances[11] and are more likely to respond to

some ovulation induction therapies than their hyperandrogenic counterparts.[85] Our study largely included women with Frank PCOS (i.e. oligo- or anovulation, hyperandrogenism, and polycystic ovarian morphology). However, two of the women had Mild PCOS (i.e. oligo- or anovulation, normal androgens, and polycystic ovarian morphology). Further studies are needed to evaluate antral folliculogenesis in normo- versus hyperandrogenic women. Finally, our study was limited by the absence of concurrent serial endocrine evaluation. Pituitary gonadotropin and ovarian steroid hormone profiles over the anovulatory interval would greatly inform the etiology of follicular emergence and turnover in PCOS**.** These assessments would also help to elucidate the mechanisms that regulate follicular growth and the spectrum of disordered folliculogenesis observed in our participants.

In summary, the observation of frequent follicular turnover in polycystic ovaries provides a new model for antral folliculogenesis in PCOS. We anticipate that knowledge of a spectrum of disordered follicle growth will have profound implications for the treatment of infertility in this condition. In particular, it may help us to understand differences in the ovulatory response to lifestyle[82] or pharmacologic interventions.[5] Consideration of these findings may also enable the identification of better predictive markers of response to these therapies in PCOS, which are necessary to guide treatment strategies and provide patients with realistic expectations for treatment outcomes.

CHAPTER 2

SONOGRAPHIC EVALUATION OF SPORADIC OVULATORY CYCLES

IN OLIGOMENORRHEIC WOMEN WITH POLYCYSTIC OVARY SYNDROME

ABSTRACT

Objectives. The extent to which antral follicle development is impaired in polycystic ovary syndrome (PCOS) is unknown. Although anovulation is a common outcome, some follicles in polycystic ovaries can occasionally progress to dominance and ovulate during natural cycles. Factors associated with the sporadic emergence of an ovulatory follicle have never been described in PCOS, largely because it is challenging to predict such an event in women with menstrual irregularity. By chance, we observed an ovulation in a subgroup of women with anovulatory phenotypes of PCOS. The objectives of the present analysis were to evaluate the growth kinetics of ovulatory follicles and identify the clinical factors associated with sporadic ovulation in PCOS.

Methods. Women with PCOS (n = 24) and Controls (n = 11) were evaluated by serial ovarian ultrasonography for 4–5 weeks. Endocrine and metabolic tests were performed on a single day during the early follicular phase. Women with PCOS were divided into two groups based on sonographic evidence of anovulation (PCOS-Anov) or sporadic ovulation (PCOS-Ov). Unique growth profiles of ovulatory follicles were assessed using offline image analysis, and the Identity Method. Diagnostic, morphologic, endocrine, and metabolic features were compared between PCOS-Anov, PCOS-Ov, and Control groups using t-tests or one-way ANOVA.

Results. Half of women with PCOS exhibited sporadic ovulation during the study. On average, ovulatory follicles were selected after shorter growth phases (6 vs. 8 days; P<0.01) and at smaller

diameters (8 vs. 10 mm; *P*=0.04) in the PCOS-Ov versus Control group. Women with PCOS-Ov were distinguished from their anovulatory counterparts by shorter cycle lengths (75 vs. 197 days; *P*=0.01), lower total testosterone concentrations (44.6 vs. 67.8 ng/dl; *P*=0.04), and lower free androgen indices (6 vs. 11; *P*=0.01). Women with PCOS-Ov had similar antral follicle counts, ovarian volumes, waist circumferences, and waist-to-hip ratios to both women with PCOS-Anov and Controls.

Conclusions. Ovulatory follicle growth kinetics are altered in women with PCOS. The implications of early follicular selection for oocyte quality, luteal function, and fertility/fecundity are unclear. Likelihood of sporadic ovulation may be linked to the severity of reproductive and endocrine disturbances in women with PCOS.

INTRODUCTION

Ovulatory dysfunction in polycystic ovary syndrome (PCOS) is characterized by the arrest of ovarian follicle development at the mid-antral stage.[47,46] Systemic endocrine and metabolic abnormalities presumably converge at the ovary[58,63,49,86] and interfere with the cyclic processes of selection and ovulation.[46] However, follicular arrest may not provide a complete explanation for the disordered folliculogenesis and anovulation in PCOS.[47,46] Namely, not all follicles in polycystic ovaries show abnormal function[58] and some are able to advance periodically towards ovulation.[87] The notion that a dominant follicle can “escape” from any systemic or intraovarian inhibition during natural cycles has never been prospectively explored in PCOS.

Oligo-ovulatory women with PCOS consistently demonstrate abnormal endocrine profiles, including elevated serum concentrations of luteinizing hormone (LH) and androgens, and reduced serum concentrations of follicle-stimulating hormone (FSH).[51,52] These profiles arise from disruptions across the hypothalamic-pituitary-ovarian axis. Pulsatile secretion of gonadotropin-releasing hormone from the hypothalamus is persistent and rapid in PCOS. This pattern favors the synthesis of LH by the anterior pituitary and contributes to an increased LH: FSH ratio.[48] High levels of LH[49] and increased activity of steroidogenic enzymes[50] augment ovarian androgen production in polycystic ovaries. Androgens may then stimulate excessive follicular growth to the selectable stage (2–5 mm),[57] while low levels of FSH inhibit further follicle maturation and contribute to reduced serum concentrations of estradiol.[47,46] Together, milder disruptions in the endocrine environment (lower LH, lower androgens, and higher FSH) could enable dominant follicle development in PCOS.[88]

Metabolic abnormalities are also common in women with PCOS.[89] Worldwide, more than half of patients are overweight or obese.[61] and roughly two-thirds of women demonstrate insulin resistance.[90] Adiposity-induced hyperinsulinemia is intimately involved with the mechanism of anovulation in PCOS.[47,46] Insulin can act on polycystic ovaries[91] to amplify ovarian androgen production[92] and increase the response of granulosa cells to LH[63] Consequently, granulosa cells

from small- and medium-sized antral follicles (4–8 mm) are able to respond to LH.[58] This implies an early acquisition of LH receptors, which in normal ovaries does not occur until the time of selection at ~10 mm.[22,58] Premature exposure of granulosa cells to LH is believed to inhibit further follicular maturation and prevent growth beyond 8–10 mm.[58] As such, improved metabolic status (lower insulin levels and better insulin sensitivity) could also promote dominant follicle development in PCOS.[64]

The factors associated with sporadic ovulation in this population are ultimately unknown. We recently performed a prospective evaluation of antral follicle development during natural cycles in PCOS (Chapter 1). Based on their histories of oligo- or amenorrhea, we expected that 85–100% of women with PCOS would remain anovulatory during study.[64] However, a substantial proportion exhibited sonographic evidence of sporadic ovulation. These serendipitous findings provided a rare opportunity to gain insight into the mechanism(s) of natural ovulation in PCOS. To that end, the objective of the present study was to evaluate follicular, endocrine, and metabolic features during sporadic ovulatory cycles in PCOS. Given the demonstration that antral follicle function[58] and growth dynamics (Chapter 1) are altered in PCOS, we hypothesized that differences in ovulatory follicle growth would also be observed in women with PCOS compared to regular ovulatory cycles. We further hypothesized that milder endocrine and metabolic abnormalities would be detected in women that experienced sporadic ovulation versus those that remained anovulatory during the study.

METHODS

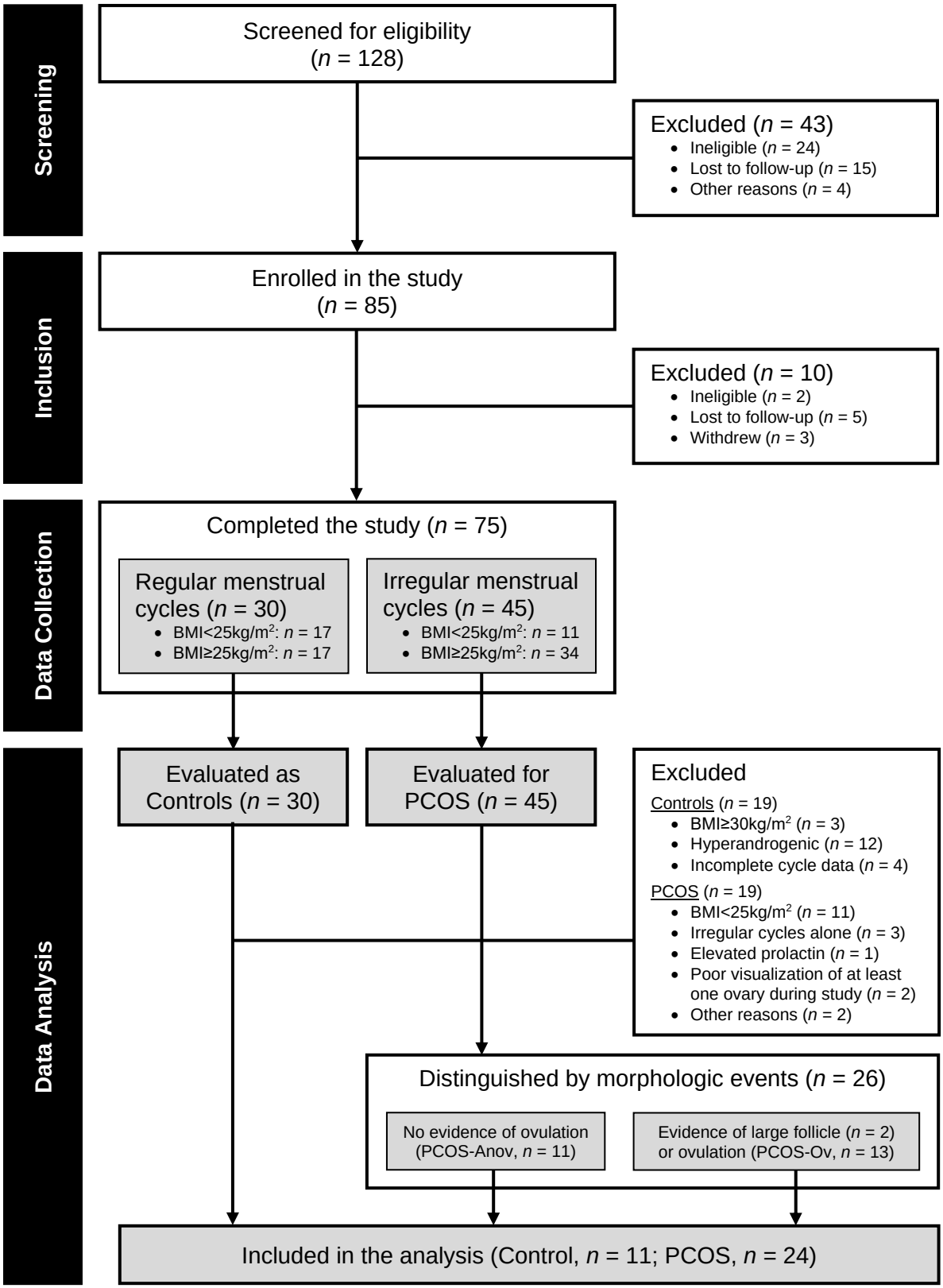
Ethical Considerations

Data from two separate, prospective, ongoing clinical trials were evaluated in this study (ClinicalTrials.gov Identifiers: NCT01927432, NCT01785719). Both research protocols were approved by the Institutional Review Board at Cornell University, and informed consent was obtained from all participants before study procedures were initiated.

Study Participants

Seventy-five women completed the study procedures (Chapter 1 and Figure 2.1). Women of reproductive age (18–38 years) were recruited through public advertisement. Exclusion criteria included the use of confounding medications in the preceding two months (e.g. hormonal contraception, fertility medications, antibiotics, antivirals), recent pregnancy or lactation, history of premature ovarian failure or surgery, and concurrent endocrine disease(s) or disorder(s) besides PCOS (e.g. thyroid abnormalities, hyperprolactinemia, pre-diabetes, diabetes mellitus). Women were evaluated for inclusion in the present analysis based on PCOS diagnosis, body mass index (BMI), and observations made during the study (Figure 2.1). Cohorts of interest included: (1) overweight / obese women with anovulatory phenotypes of PCOS (BMI ≥25.0 kg/m^2) and (2) normal- / overweight (BMI 18.5–29.9 kg/m^2) women with regular ovulatory cycles (Control) (Chapter 1). Women with PCOS were further stratified based on ultrasonographic evidence of anovulation (PCOS-Anov) or sporadic ovulation (PCOS-Ov) during the study (Figure 2.1). PCOS was diagnosed according to the Rotterdam criteria,[8,9,19] as previously described (Chapter 1). By definition, all women in the PCOS-Anov and PCOS-Ov groups were oligo- or anovulatory at the time of enrollment, based on self-report of amenorrhea (no bleeding for the past 3 months) or oligomenorrhea (fewer than 10 periods in the past year).

Figure 2.1. Flow of participants through the observational cohort study (2009–2016) and present analysis. *Shaded boxes designate the cohorts from which participants were selected for the present analysis. Abbreviations: PCOS, polycystic ovary syndrome; BMI, body mass index.*



Study Design

The study was conducted over a period of 4–6 weeks. Participants visited the research unit every other day during the study. At each visit, transvaginal ultrasonography of the ovaries was performed to evaluate follicle growth (Chapter 1). Women underwent additional clinical and biochemical assessments at one of the visits to measure endocrine and metabolic status. Details of ultrasonographic, clinical, and biochemical assessments are provided below.

Ultrasonographic Measurements

Ultrasound scanning regimens differed between groups. In women with PCOS, scans were initiated at a random time and continued every other day for the duration of the study. In Controls, scans began on days 8–14 after menses and were performed every other day for one inter-ovulatory interval (IOI). An IOI was defined as the time between consecutive ovulations.[44,72] Follicular growth was closely monitored in real-time. If a large follicle (≥14 mm) was detected, then ultrasound examinations were performed every day until its regression or ovulation. Ovulation was defined by disappearance of the large follicle and subsequent formation of a corpus luteum.[73,74] Anovulation was defined by an absence of follicle growth >10 mm. Ovarian data were acquired with a high-resolution ultrasound machine and 6–12 MHz endovaginal transducer (GE Voluson E8 Expert System, GE Healthcare, Milwaukee, WI). Whole ovaries were imaged from their inner to outer margins in the longitudinal plane, and digital cineloops were recorded for offline analysis (Santesoft DICOM Editor, Santesoft LTD, Athens, Greece).

Follicles were counted and measured in each cineloop using the grid system approach.[75] Data from the left and right ovaries were combined. The numbers of follicles detected across the scanning interval were then binned into five diameter categories: ≥2 mm, 2–9 mm, 2–5 mm, 6–9 mm, and ≥10 mm. Mean ovarian volume was also calculated at each visit using the formula for a prolate ellipsoid.[20,93,94]

Methods used for tracking follicle diameter over time were performed as described in Chapter 1. In the present analysis, growth trajectories of ovulatory follicles were characterized from emergence to ovulation. Day of emergence was defined as the day on which the ovulatory follicle was first detected, retrospectively, at 4–5 mm.[43] Day of selection was defined as the day immediately preceding a difference in growth trajectories between the ovulatory follicle and other follicles present in the ovaries. Specifically, the ovulatory follicle continued to grow, while the next largest follicle(s) began a static or regression phase.[72] An ovulatory follicle was considered dominant when it grew to ≥10 mm and exceeded the diameters of other follicle(s) by at least 2 mm.[43] The interval of time and growth rate of the ovulatory follicle between each event was determined (Chapter 1).

Clinical and Biochemical Measurements

The following procedures were conducted in the morning after an overnight fast: (1) hirsutism assessment, (2) anthropometry, (3) dual x-ray absorptiometry, (4) venipuncture, and (5) 2-hour 75-gram oral glucose tolerance test. In both the PCOS and Control groups, tests were standardized to the early follicular phase, on a day when no dominant follicles or active corpora lutea were present. Male-patterned hair growth was evaluated using the modified Ferriman-Gallwey scoring system[95], as previously described[65]. Anthropometry was performed to assess height, weight, and waist circumference. Participants were weighed with light clothes and no shoes on a standard digital scale; BMI was calculated as weight (kilograms) divided by squared height (meters). Waist circumference was measured with soft tape at the midpoint between the lowest rib and iliac crest. Dual x-ray absorptiometry (Discovery-A, Hologic, Inc., Bedford, MA) was performed to assess total and abdominal body fat. Total fat was determined by measurement of fat versus lean mass in soft tissue, and abdominal fat was distinguished as the region between the ribs and iliac crests.

Fasting concentrations of total testosterone and sex hormone binding globulin (SHBG) were measured, as previously described (Chapter 1). The free androgen index was calculated (testosterone / SHBG × 100) and used as a surrogate estimate of free testosterone. Serum concentrations of FSH, LH, and estradiol were measured by chemiluminescent immunoassay (Immulite 2000; Siemens Medical Solutions Diagnostics, Deerfield, IL). Intra- and inter-assay coefficients of variation were as follows: FSH and LH (3.4%, 5.4%), and estradiol (6.7%, 9.7%). Glucose and insulin concentrations were assessed at 0, 30, 60, 90, and 120 minutes of the oral glucose tolerance test. Venous glucose was immediately quantified with a glucometer (Accu-Check® Aviva, Roche Diabetes Care, Inc., Indianapolis, IN). Serum was isolated from whole blood (Chapter 1). Insulin was measured by chemiluminescent immunoassay (Immulite 2000; Siemens Medical Solutions Diagnostics, Deerfield, IL). Intra- and inter-assay coefficients of variation for insulin were 4.8% and 6.2%, respectively. The homeostatic model assessment of insulin resistance (HOMA) and whole-body insulin sensitivity index (WBISI) were calculated as surrogate markers of insulin sensitivity.[96]

Statistical Analysis

Statistical analyses were performed with JMP Pro 12.0.1. (SAS Institute, Cary, NC, USA). The threshold for significance was set at $P \leq 0.05$. Profiles of follicle number and diameter were centralized to the day of the first study visit (PCOS) or ovulation (Control), and data were averaged over the entire scanning interval. Normality was assessed visually with histograms and skewed data were log-transformed for between-group comparisons where appropriate. Women with PCOS were considered as a combined cohort (“PCOS”) and as two separate groups (“PCOS-Anov” and “PCOS-Ov”). Differences between groups were evaluated by two-sample t-tests or one-way ANOVA with post-hoc Tukey’s HSD tests. One subject was taking an insulin-sensitizing medication during the study. The medication had been prescribed for PCOS; she did not have

pre-diabetes or diabetes mellitus. Sensitivity analyses confirmed that her inclusion in the dataset did not have any impact on the results.

RESULTS

Characteristics of Study Participants

Study enrollment is described in Figure 2.1. Complete datasets were available for 26 overweight and obese women with anovulatory phenotypes of PCOS. Eleven of the 26 women with PCOS (42%) exhibited anovulation (PCOS-Anov) during the study and 13 (50%) showed sonographic evidence of sporadic ovulation (PCOS-Ov). Dominant follicles (≥14 mm) were detected in two additional women (8%), but the study ended before their fates could be determined. Therefore, both women were excluded from the present analysis. Results are presented for 24 overweight or obese women with PCOS and 11 non-obese women with regular ovulatory cycles. Diagnostic features are compared between PCOS and Controls in Table 2.1.

Table 2.1. Characteristics of study participants

	PCOS (*n* = 24)	**Control (*n* = 11)**
Age (y)	28 ± 5 (21, 36)[a]	29 ± 6 (19, 38)[a]
Body mass index (kg/m^2)	35.5 ± 7.0 (25.7, 48.4)[a]	23.9 ± 1.9 (21.2, 27.3)[b]
Menstrual cycle length (d)	136 ± 118 (41, 365)[a‡]	29 ± 2 (24, 31)[b]
Hirsutism score	7 ± 4 (1, 15)[a]	2 ± 3 (0, 6)[b]
Total testosterone (ng/dL)	55.7 ± 26.7 (15.8, 118.0)[a]	30.5 ± 9.6 (17.6, 43.6)[b]
Free androgen index	8 ± 5 (1, 23)[a]	2 ± 1 (1, 3)[b]
Mean follicle number per ovary	43 ± 21 (14, 84)[a]	26 ± 7 (15, 36)[b]
Mean ovarian volume (mL)	14 ± 5 (7, 21)[a]	6 ± 4 (2, 12)[b]

Data are presented as mean ± SD (minimum, maximum). [a,b] Within rows, uncommon superscripts denote significant differences between groups determined by t-test, $p<0.05$. Diagnostic endpoints were evaluated on a single day of the scanning interval and with respect to stage of cycle. [‡] Data related to menstrual cycle length were censored to the year prior to enrollment (i.e. 365 days).

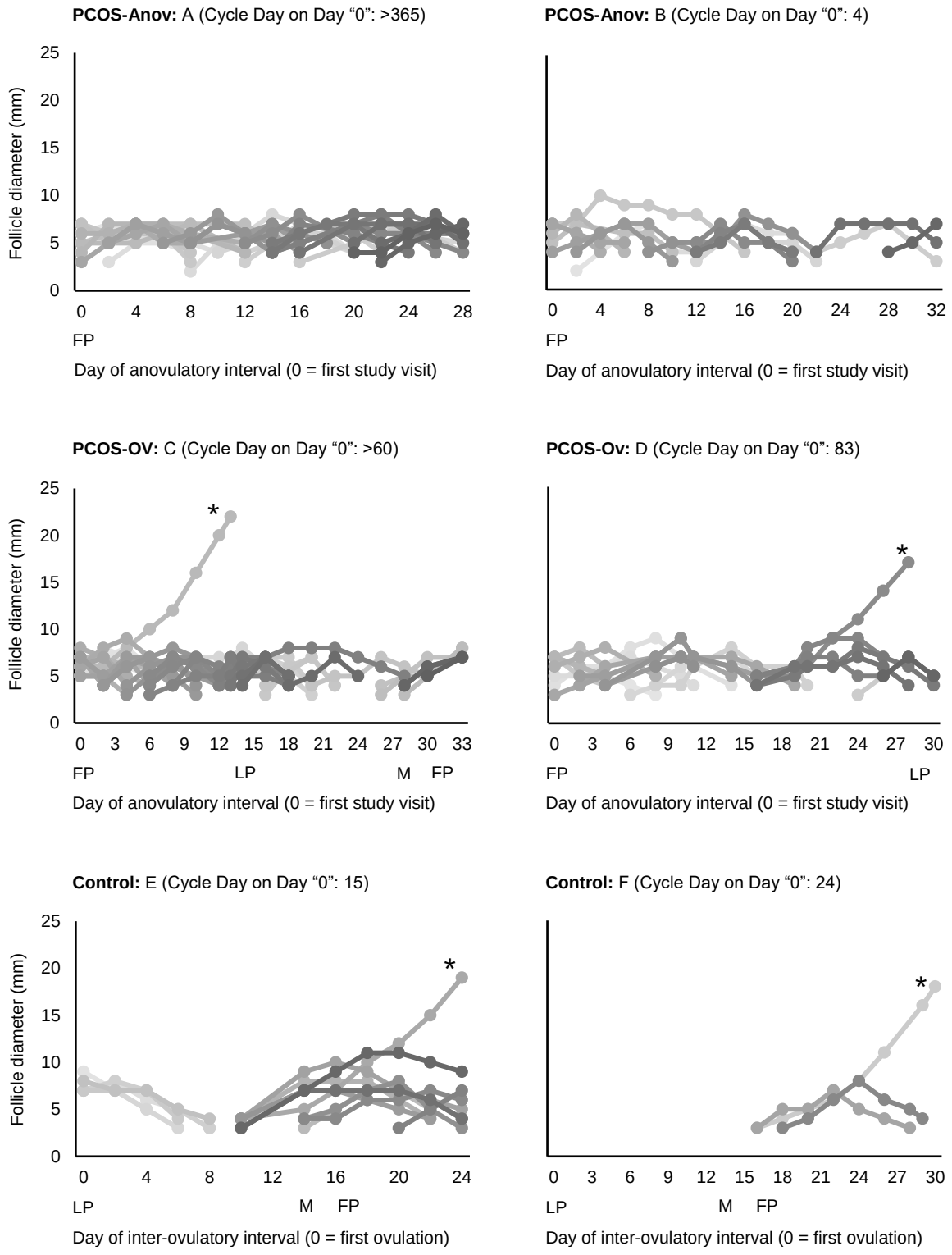
Observation of Sporadic Ovulation

Women with PCOS (*n*=24) reported having a maximum of nine periods in the year prior to enrollment (Table 2.1). Last menses had occurred approximately two months before the first study visit (mean ± SD: 54 ± 80 days; range: 4 to >365 days). Representative plots of follicle growth during the scanning interval are illustrated for the PCOS-Anov, PCOS-Ov, and Control groups in Figure 2.2.

Women with PCOS-Anov (*n*=11) were evaluated for a mean of 30 ± 5 days. Follicular growth did not exceed 10 mm at any time during the scanning interval (Figure 2.2, A–B). The majority of uniquely identified follicles developed to a mean diameter of 7.2 ± 0.2 mm before undergoing atresia (Chapter 1; Figure 2.2, A). Three of the 11 women with PCOS-Anov showed evidence of dominance despite anovulation. Each dominant follicle (*n*=3/3) emerged at the 4–5 mm stage, developed to a maximum diameter of 10 mm, and then regressed 1–2 days later (Figure 2.2, B).

Women with PCOS-Ov (*n*=13) were evaluated for a mean of 31 ± 5 days. Ovulation was observed once in each participant (Figure 2.2, C–D). Ovulatory follicles either emerged before the first study visit (*n*=4/13; Figure 2.2, C) or between the first and third weeks of the scanning interval (*n*=9/13; Figure 2.2, D). Ovulatory follicles that emerged before the first study visit were tracked from selection (*n*=3/13) or pre-ovulatory diameters (*n*=1/13) to ovulation (Figure 2.2, C). Ovulatory follicles that emerged between the first and third weeks of the scanning interval were tracked from emergence to ovulation (*n*=9/13; Figure 2.2, D). Consistent with the criteria for oligo- or amenorrhea, mean follicular phase length from self-reported last menses to ovulation was 56 ± 45 days (PCOS-Ov, *n*=13). A complete luteal phase was documented in 11 of 13 women (mean luteal phase length ± SD: 12 ± 2 days). The remaining two women left the study after ovulation had been observed, but before the subsequent menses began.

Figure 2.2. Representative plots of follicle growth during the scanning interval from women in the PCOS-Anov (*n* = 2; A–B), PCOS-Ov (*n* = 2; C–D), Control (*n* = 2; E–F) groups. *Each uniquely identified follicle (that grew to ≥7 mm) is represented by a different gray line. Asterisks indicate the ovulatory follicles. Abbreviations: FP, follicular phase; LP, luteal phase; M, menses.*



Follicle Growth

Growth kinetics of ovulatory follicles are compared between the PCOS-Ov and Control groups in Table 2.2. Overall, the mean growth phase (*P*=0.90) and growth rate (*P*=0.98) of ovulatory follicles from emergence to ovulation did not differ in women with PCOS-Ov versus Controls. However, differences were noted between the two groups before and after selection. The mean interval from emergence to selection was shorter in women with PCOS-Ov than in controls (*P*<0.01). This resulted in a smaller mean diameter on the day of selection in the PCOS-Ov group (8 versus 10 mm, respectively; *P*=0.04). Conversely, the mean interval from selection to ovulation was longer in women with PCOS-Ov than in Controls (*P*<0.01). This resulted in a slightly larger maximum pre-ovulatory diameter in the PCOS-Ov group (20 versus 19 mm; *P*=0.09). Luteal phase lengths did not differ between groups (*P*=0.38) (Table 2.2).

Table 2.2. Growth characteristics of ovulatory follicles in the PCOS-Ov and Control groups

	PCOS-Ov (*n* = 13)	**Control (*n* = 11)**
Emergence to ovulation		
Growth phase (d)	14 ± 3 (11, 20)[a]	14 ± 2 (8, 17)[a]
Growth rate (mm/d)	1.10 ± 0.27 (0.85, 1.64)[a]	1.10 ± 0.20 (0.82, 1.50)[a]
Emergence to selection		
Growth phase (d)	6 ± 2 (3, 7)[a]	8 ± 2 (5, 13)[b]
Growth rate (mm/d)	0.66 ± 0.19 (0.40, 1.00)[a]	0.73 ± 0.21 (0.50, 1.11)[a]
Diameter at selection (mm)	8 ± 1 (7, 10)[a]	10 ± 2 (7, 14)[b]
Selection to ovulation		
Growth phase (d)	10 ± 3 (5, 17)[a]	7 ± 2 (3, 10)[b]
Growth rate (mm/d)	1.25 ± 0.30 (0.82, 1.78)[a]	1.39 ± 0.34 (1.00, 2.25)[a]
Maximum diameter (mm)	20 ± 2 (16, 23)[a]	19 ± 2 (16, 23)[a]
Luteal phase length (d)	12 ± 2 (7, 15)[a]	13 ± 1 (11, 15)[a]

Data are presented as mean ± SD (minimum, maximum). [a,b] Within rows, uncommon superscripts denote significant differences between groups determined by t-test, p<0.05. Each woman contributed data from a single ovulatory follicle.

Between-Group Differences in Diagnostic Features

Diagnostic features are compared among the PCOS-Anov, PCOS-Ov, and Control groups in Table 2.3. By design, women with PCOS differed from Controls by longer menstrual cycles (PCOS-Anov: $P<0.01$; PCOS-Ov: $P=0.01$), higher hirsutism scores (PCOS-Anov: $P<0.01$; PCOS-Ov: $P=0.03$), higher free androgen indices (PCOS-Anov: $P<0.01$; PCOS-Ov: $P<0.01$), and larger ovarian volumes (PCOS-Anov: $P<0.01$; PCOS-Ov: $P<0.01$). Women with PCOS-Anov were further distinguished from Controls by higher total testosterone concentrations ($P<0.01$) and higher mean follicle counts ($P=0.01$). Conversely, women with PCOS-Ov showed similar total testosterone concentrations ($P=0.21$) and mean follicle counts ($P=0.34$) to Controls. They also had shorter menstrual cycle lengths ($P=0.01$), lower total testosterone concentrations ($P=0.04$), and lower free androgen indices ($P=0.01$) compared to the PCOS-Ov group (Table 2.3).

Between-Group Differences in Ovarian Morphology

Follicle diameter populations (2–9 mm, 2–5 mm, 6–9 mm) and mean ovarian volumes were averaged over the entire scanning interval and are shown for the PCOS-Anov, PCOS-Ov, and Control groups in Figure 2.3. Women with PCOS-Anov were distinguished from Controls by higher numbers of total (2–9 mm; $P<0.01$) and small follicles (2–5 mm; $P=0.01$), as well as larger ovarian volumes ($P=0.03$). Conversely, women with PCOS-Ov showed similar follicle counts and ovarian volumes to both women with PCOS-Anov and Controls. Mean numbers of medium-sized follicles (6–9 mm) did not differ across groups (Overall: $P=0.13$) (Figure 2.3).

Between-Group Differences in Correlates of Aberrant Folliculogenesis

Endocrine and metabolic features are compared among the PCOS-Anov, PCOS-Ov, and Control groups in Table 2.4. Mean concentrations of pituitary and ovarian hormones did not differ across groups (overall for all hormones: $P>0.05$). Consistent with the inclusion criteria for the present analysis, women with PCOS were largely obese, and Controls were normal weight (BMI

<25.0 kg/m^2). Women with PCOS also demonstrated greater total and abdominal adiposity than Controls (both features, PCOS-Anov versus Control: *P*<0.01; PCOS-Ov versus Control: *P*<0.01), and waist circumference (*P*<0.01) and waist-to-hip ratio (*P*=0.02) differed between the two groups. Women with PCOS showed higher fasting insulin concentrations (PCOS-Anov versus Control: *P*<0.01; PCOS-Ov versus Control: *P*=0.04), higher HOMA-IR values (PCOS-Anov versus Control: *P*<0.01; PCOS-Ov versus Control: *P*=0.02), and lower WBISI values compared to Controls (PCOS-Anov versus Control: *P*<0.01; PCOS-Ov versus Control: *P*<0.01) (Table 2.4). Overall, the PCOS-Anov and PCOS-Ov groups did not differ in any of the endocrine or metabolic features evaluated (Table 2.4).

Table 2.3. Differences in diagnostic features between the PCOS-Anov, PCOS-Ov, and Control groups

	PCOS-Anov (*n* = 11)	**PCOS-Ov** (*n* = 13)	**Controls** (*n* = 11)
Menstrual cycle length (d)	197 ± 138 (50, 365)[a‡]	75 ± 44 (41, 180)[b‡]	29 ± 2 (24, 31)[c]
Hirsutism score	8 ± 4 (1, 15)[a]	6 ± 4 (2, 12)[a]	2 ± 3 (0, 6)[b]
Total testosterone (ng/dl)	67.8 ± 27.9 (32.9, 118.0)[a]	44.6 ± 21.0 (15.8, 81.5)[b]	30.5 ± 9.6 (17.6, 43.6)[b]
Free androgen index	11 ± 6 (4, 23)[a]	6 ± 3 (1, 15)[b]	2 ± 1 (1, 3)[c]
Mean follicle number per ovary	50 ± 22 (14, 80)[a]	37 ± 20 (14, 84)[ab]	26 ± 7 (15, 36)[b]
Mean ovarian volume (ml)	16 ± 4 (7, 21)[a]	12 ± 5 (7, 20)[a]	6 ± 4 (2, 12)[b]

Data are presented as mean ± SD (minimum, maximum). [a,b,c] Within rows, uncommon superscripts denote significant differences between groups determined by one-way ANOVA with post-hoc Tukey's HSD test, $p<0.05$. Diagnostic endpoints were evaluated on a single day of the scanning interval and with respect to stage of cycle. [‡] Data related to menstrual cycle length were censored to the year prior to enrollment (i.e. 365 days).

Figure 2.3. Mean values of sonographic endpoints during the entire scanning interval for women in the PCOS-Anov, PCOS-OV, and Control groups. *Box-and-whisker plots are shown for three follicle size populations (2–9 mm, 2–5 mm, 6–9 mm) and ovarian volume. The box represents the 25th and 75th percentiles and the horizontal line within the box represents the median. The 5th to 95th percentile range is reflected by the vertical bars. Outliers, if any, are denoted by dots. Uncommon letters denote significance differences between groups, p<0.05.*

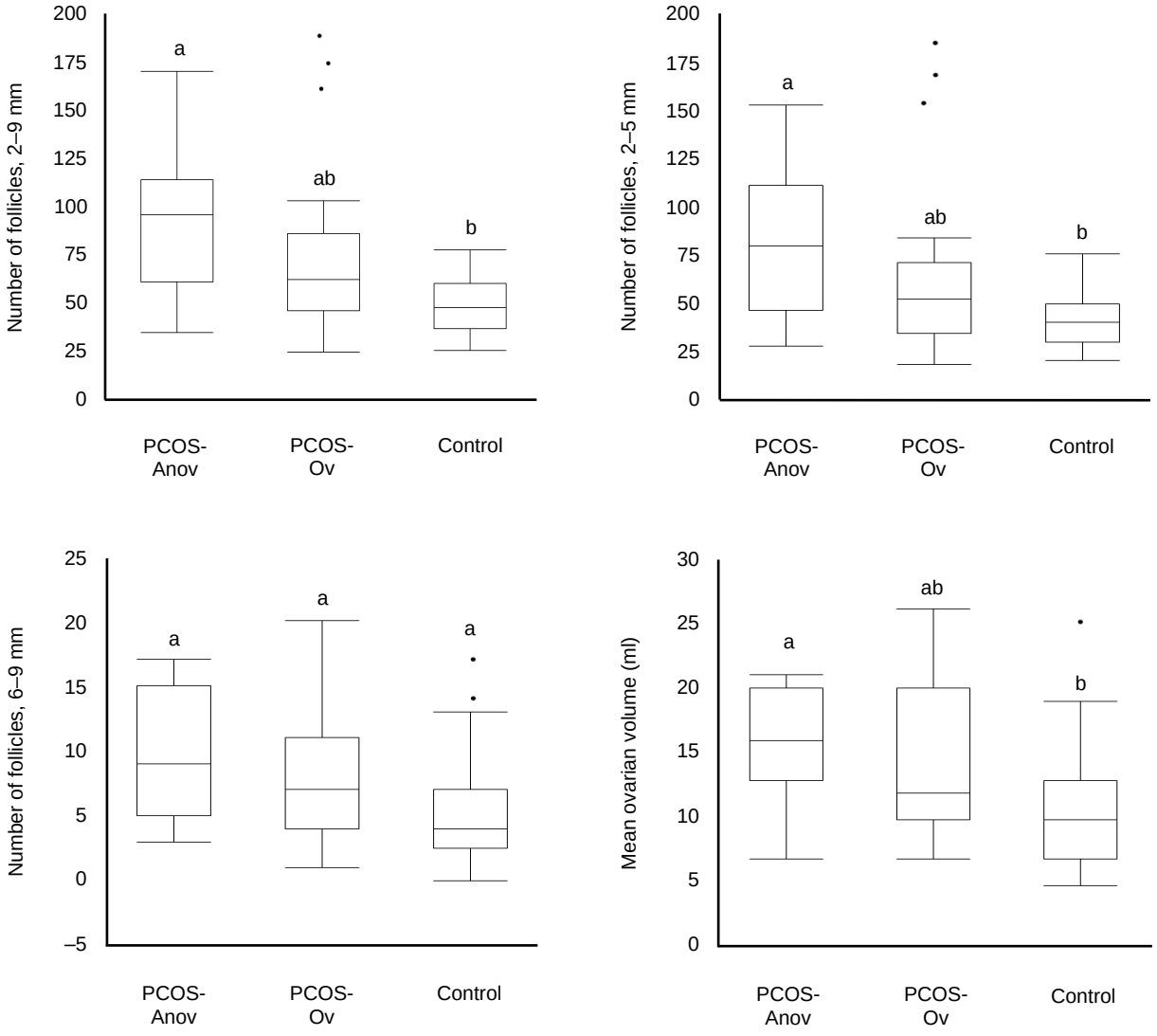


Table 2.4. Differences in correlates of aberrant folliculogenesis between the PCOS-Anov, PCOS-Ov, and Control groups

	PCOS-Anov (*n* = 11)	**PCOS-Ov** (*n* = 13)	**Controls** (*n* = 11)
Endocrine			
FSH (mIU/ml)	6.0 ± 1.9 (2.7, 9.2)[a]	6.5 ± 2.0 (2.7, 9.4)[a]	8.1 ± 2.2 (5.2, 12.3)[a]
LH (mIU/ml)	7.4 ± 2.2 (3.0, 9.7)[a]	6.9 ± 3.6 (1.6, 14.3)[a]	4.8 ± 2.0 (2.0, 8.3)[a]
Estradiol (pg/ml)	59.4 ± 19.7 (28.2, 94.8)[a]	56.7 ± 23.6 (24.8, 104.0)[a]	42.5 ± 25.0 (26.8, 97.9)[a]
Metabolic			
BMI (kg/m^2)	36.3 ± 8.0 (26.5, 48.4)[a]	34.8 ± 6.2 (25.7, 46.2)[a]	23.9 ± 1.9 (21.2, 27.3)[b]
WC (cm)	109 ± 23 (76, 157)[a]	99 ± 16 (71, 127)[ab]	82.3 ± 7.2 (73, 94)[b]
Waist-to-hips ratio	0.91 ± 0.09 (0.76, 1.08)[a]	0.86 ± 0.06 (0.75, 0.98)[ab]	0.81 ± 0.05 (0.73, 0.88)[b]
Body fat (%)	40 ± 6 (30, 48)[a]	41 ± 6 (31, 46)[a]	28 ± 6 (19, 37)[b]
Abdominal fat (%)	42 ± 7 (32, 52)[a]	40 ± 8 (25, 50)[a]	25 ± 7 (15, 37)[b]
Fasting insulin (mIU/ml)	21.6 ± 18.9 (2.2, 56.1)[a]	12.3 ± 4.9 (7.1, 22.0)[a]	6.0 ± 3.8 (2.0, 14.3)[b]
HOMA-IR	5.5 ± 4.8 (0.5, 14.7)[a]	3.0 ± 1.2 (1.6, 5.4)[a]	1.4 ± 0.8 (0.5, 2.6)[b]
WBISI	4.4 ± 4.8 (0.6, 15.7)[a]	3.9 ± 1.8 (1.6, 7.4)[a]	12.7 ± 5.6 (6.2, 21.4)[b]

Data are presented as mean ± SD (minimum, maximum). [a,b] Within rows, uncommon superscripts denote significant differences between groups determined by one-way ANOVA with post-hoc Tukey's HSD test, $p<0.05$. Metabolic and endocrine endpoints were evaluated on a single day of the scanning interval and with respect to stage of cycle. *Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; BMI; body mass index; WC, waist circumference; HOMA-IR, homeostatic model assessment of insulin resistance; WBISI, whole-body insulin sensitivity index.*

DISCUSSION

Our data are unique, in that they provide the first-ever longitudinal evidence that some follicles in polycystic ovaries can “escape” arrest and progress through advanced stages of maturation during natural cycles. Through comprehensive sonographic analyses, we described the trajectory of this development and identified differences in ovulatory follicle growth kinetics between women with PCOS and Controls. We also found that milder reproductive and endocrine abnormalities were associated with sporadic ovulatory cycles versus continued anovulation. Although our study was not designed to appreciate the implications of this altered growth in ovulatory follicles fully, our data do raise important questions regarding the actual relevance and/or health benefits of sporadic cycles in women with PCOS.

Ovulatory follicles were selected after shorter growth phases and at smaller diameters in women with PCOS-Ov compared to Controls. To the best of our knowledge, early selection has never been reported during natural or induced cycles in PCOS. This novel finding supports the notion that granulosa cells from polycystic ovaries prematurely acquire LH receptors.[58] The theory of follicular arrest suggests that inappropriate exposure to LH can suppress granulosa cell proliferation and initiate atresia in non-dominant follicles.[58,32] Yet, evidence of dominance and ovulation in our cohort indicates that some follicles can be “rescued” from arrest. Ovulatory follicles in normal ovaries are believed to have early morphologic and functional advantages over other subordinate follicles.[22] It might be speculated that follicles that progress to ovulation in polycystic ovaries have more granulosa cells and FSH receptors than follicles that are destined for arrest. This might result in an increased ability to produce estradiol[86] and/or escape the detrimental effects of excessive and untimely LH exposure. That being said, these endocrine abnormalities have been linked to impaired oocyte development, failure of implantation, and miscarriage in PCOS.[97,98] Rescuing a follicle from arrest may, therefore, have negative implications for oogenesis, luteal function, and endometrial receptivity.

Women with PCOS-Ov exhibited longer intervals from selection to ovulation and had slightly larger pre-ovulatory follicle diameters than Controls, but the significance of delayed ovulation in PCOS remains to be determined. Ovulatory follicle growth rates and pre-ovulatory diameters in the PCOS-Ov group were in line with ranges that have been reported in healthy women during natural cycles.[43,45] Therefore, longer growth phases from selection to ovulation likely reflected earlier selection alone, since overall growth intervals did not differ between women with PCOS-Ov and Controls. Because the timing of ovulation depends on a hormonal interplay between the hypothalamus, pituitary, and ovary, comprehensive endocrine profiles during the periovulatory period are needed to fully appreciate the degree to which ovulatory events differed between groups.

Milder reproductive features were associated with sporadic ovulation in PCOS. Women with PCOS-Ov had shorter cycles, lower total testosterone, and lower free androgen indices than their anovulatory counterparts. Mean hirsutism scores, follicle number per ovary, and ovarian volume were also intermediate to women with PCOS-Anov and Controls. The observation that women with anovulatory intervals were largely hyperandrogenic supports the notion that androgens are a main driver of abnormal follicular maturation in PCOS.[47] Similarly, the observation that women with sporadic ovulatory cycles were largely normoandrogenic (62%) supports the notion of an inverse association between androgens and degree of reproductive disturbance.[88] We anticipated greater differences in endocrine and metabolic features between the PCOS-Anov and PCOS-Ov groups. Gonadotropins and markers of insulin sensitivity were intermediate in women with PCOS-Ov compared to women with PCOS-Anov and Controls, but differences did not reach significance. The theory that milder endocrine and metabolic disturbances can enable aspects of “normal” folliculogenesis in PCOS is consistent with our previous studies showing a higher incidence of dominant follicles in women with better insulin sensitivity[64] and the inverse association between androgens and follicle number.[88] It is likely that we did not detect differences in endocrine and metabolic status between groups, because women

with PCOS-Ov still represented a severe phenotype of the condition. Sporadic ovulation was a chance finding and was not indicative of normal ovulatory status. Cross-sectional assessments of clinical features may not have been sufficient to capture more transient improvements in endocrine and metabolic health. In the same way, our sample size (PCOS, *n* = 24) was sufficient to fulfill our primary objective of describing follicle growth kinetics over time (α = 0.05, power = 80%). However, this analysis was largely serendipitous and not powered to detect smaller endocrine or metabolic differences between PCOS-Anov and PCOS-Ov.

This study's strengths lie in the assessment of growth dynamics of individual identified ovulatory follicles over time. Serial changes in follicle diameter were captured in the context of follicular excess and compared with mean outcomes and clinical features over the scanning interval. However, we acknowledge that interpretation of these findings is limited by the lack of concurrent serial endocrine data. Additional studies are required to determine the associations between altered follicle growth kinetics and serial changes in gonadotropins and ovarian steroids. Such assessments will enable more discrete assessments of follicular and luteal function and are ultimately needed to capture any impact/health benefits of sporadic ovulation in PCOS. Chronic anovulation alone is associated with osteoporosis,[99] cardiovascular disease,[100] and gynecologic cancers.[101] Having sporadic ovulations may, therefore, reduce the risk for these poor health outcomes. Similarly, women with ovulatory phenotypes of PCOS are expected to have lower morbidity and mortality across the lifespan, though longitudinal data are still needed.[102]

In summary, ovulatory follicle growth kinetics are altered during sporadic ovulatory cycles in PCOS. Future assessment of these data, in conjunction with endocrine dynamics, will enable a better understanding of the mechanisms that govern oocyte development, luteal function, and endometrial receptivity in PCOS. Androgen excess may be an important target for interventions aimed at reinstating ovulation in this population.

CHAPTER 3

UTILITY OF THE UPDATED ULTRASOUND CRITERIA FOR POLYCYSTIC OVARY SYNDROME ACROSS THE MENSTRUAL CYCLE

ABSTRACT

Objectives. Diagnosis of polycystic ovary syndrome (PCOS) requires sonographic evaluation of polycystic ovarian morphology (PCOM) during the early follicular phase of a natural or induced cycle. This approach is based on evidence that follicle number and ovarian size can fluctuate during the normal menstrual cycle. The objective of this study was to determine the impact of a dominant follicle or corpus luteum on the morphologic diagnosis of PCOS.

Methods. Twenty-six women with irregular menstrual cycles and PCOS were evaluated by serial ovarian ultrasonography for 3–6 weeks. Mean follicle number per ovary (FNPO), follicle number per cross-section (FNPS), and ovarian volume (OV) were quantified every other day throughout the study. Changes in each marker were assessed across a random anovulatory interval (PCOS-ND, n=8) and different phases of the menstrual cycle (PCOS-D, n=18).

Results. Mean values for FNPO and OV exceeded the diagnostic thresholds on each day of the study in the PCOS-ND and PCOS-D groups. FNPO remained constant throughout the random anovulatory interval (PCOS-ND, P=0.23) and menstrual cycle (PCOS-D, P=0.48). Similar to previous reports in women with regular cycles, OV increased during the luteal phase (PCOS-D, P<0.01) and resulted in more false-positive diagnoses of PCOM (P=0.06). Conversely, mean values for FNPS fell below the diagnostic threshold on most days of the study in the PCOS-ND and PCOS-D groups. FNPS was largely impacted by image quality (PCOS-ND, 30% variance) and stage of cycle (PCOS-D, P<0.01).

Conclusions. FNPO was the most robust morphologic marker of PCOS over time. Diagnostic evaluations of OV were altered by the presence of a dominant follicle and a corpus luteum, and should, therefore, be confined to the early follicular phase. FNPS was an inconsistent marker of PCOM across the menstrual cycle; its use as a surrogate for FNPO should be avoided.

INTRODUCTION

Polycystic ovarian morphology (PCOM) has long been associated with the condition of polycystic ovary syndrome (PCOS). The first description of PCOM came in 1935, when Stein and Leventhal observed bilateral polycystic ovaries in seven women with amenorrhea.[16] Decades later, three seminal studies established follicular excess and stromal hypertrophy as morphological hallmarks of the condition.[53,55,54] The application of high-resolution ultrasound technology enabled non-invasive evaluation of these features and highlighted the high prevalence of PCOM in women with anovulation or androgen excess.[14] Over time, consensus mounted in both clinical and research communities that PCOM represents an important diagnostic marker of PCOS.[6,8,9,14]

As such, reliable and accurate morphological criteria are needed to distinguish normal ovaries from polycystic ovaries.[18] This was formally appreciated in 2003, when an expert panel proposed the first diagnostic thresholds for PCOM.[8,9] These “Rotterdam criteria” identified follicle number per ovary (FNPO) and ovarian volume (OV) as accurate sonographic markers of the polycystic ovary and defined PCOM by an FNPO ≥12 follicles or OV ≥10 ml.[8,9] The panel also recommended that transvaginal approaches be used, that women be scanned at a random time or after a progestin-induced withdrawal bleed, and that the presence of a dominant follicle or corpus luteum be sufficient to delay evaluation. The thresholds were established using the only diagnostic test study available[103] and technical aspects were based on expert opinion.[8,9,20]

Dissemination of these criteria into clinical practice led to controversy over the specificity of PCOM to the condition of PCOS. Numerous reports began to suggest that the thresholds were inappropriate and contributed to a false diagnosis in >60% of healthy women.[104,105] Many clinicians and researchers argued that the clinical and biochemical criteria were paramount to detect PCOS – with little information garnered from sonographic evidence.[14,15] At the same time, others urged that the morphological criteria just needed further attention, with a comprehensive evaluation of normative data and any impact of newer imaging technology.[66,106,107] In 2012, the

National Institutes of Health prioritized these evaluations in the national research agenda for PCOS.[6]

Recently, the Androgen Excess and PCOS (AE-PCOS) Society responded with a revised definition for PCOM.[19] FNPO and OV were reaffirmed as accurate reflections of follicular excess and stromal hypertrophy, and a thorough review of available literature was conducted to identify ranges for these metrics in healthy women.[19] This process resulted in two notable changes from the Rotterdam criteria. First, the diagnostic threshold for FNPO was raised from ≥12 to ≥25 follicles. Second, the threshold for OV was maintained at ≥10 ml, but recommended use of the marker was limited to cases of poor image quality or older technology (i.e. <8MHz).[19] The panel acknowledged that FNPO had superior sensitivity to detect PCOS[66,107] and that the utility of any other common metrics (i.e., follicle number per cross-section, FNPS) remained uncertain.[19,66] Other technical aspects, related to the timing of evaluation, were not directly addressed.

Ultimately, these new thresholds are expected to improve the sonographic evaluation of PCOM.[19] However, their biological accuracy remains in question. A main issue relates to the permanence of follicular excess over time. In healthy women of reproductive age, follicle populations have been shown to fluctuate from day-to-day and between the follicular and luteal phases of the menstrual cycle.[22] Ovarian size has also been shown to increase with the growth of a dominant follicle or corpus luteum.[108] In clinical practice, these anatomical changes provide important rationale for limiting diagnostic evaluations to the early follicular phase of a natural or induced cycle.[8,9,20] However, performing multiple ultrasound scans may not be feasible in certain practices or resource-limited settings. Patients may have to travel from remote locations for care and insurance providers may only cover the cost of one diagnostic visit. Thus, the time and cost associated with additional ultrasound scans may impart a significant burden on women. Likewise, in research settings, the inconvenience of multiple scans for ensuring eligibility may hinder enrollment and retention of participants. It is worthwhile to understand whether fluctuations in follicle number or ovarian size impact the utility of the morphological criteria to detect PCOS.

Our recent evaluation of antral follicle dynamics in anovulatory (Chapter 1) and sporadic ovulatory cycles (Chapter 2) provides a unique opportunity to address this question. In the present analysis, our objective was to assess the impact of a dominant follicle or corpus luteum on the morphologic diagnosis of PCOS. Based on evidence from our diagnostic test studies, we hypothesized that FNPO would be the most robust marker of polycystic ovarian morphology over time. We further hypothesized that both FNPS and OV would be impacted by cycle phase, because these measurements are based on very limited views of the ovaries.

METHODS

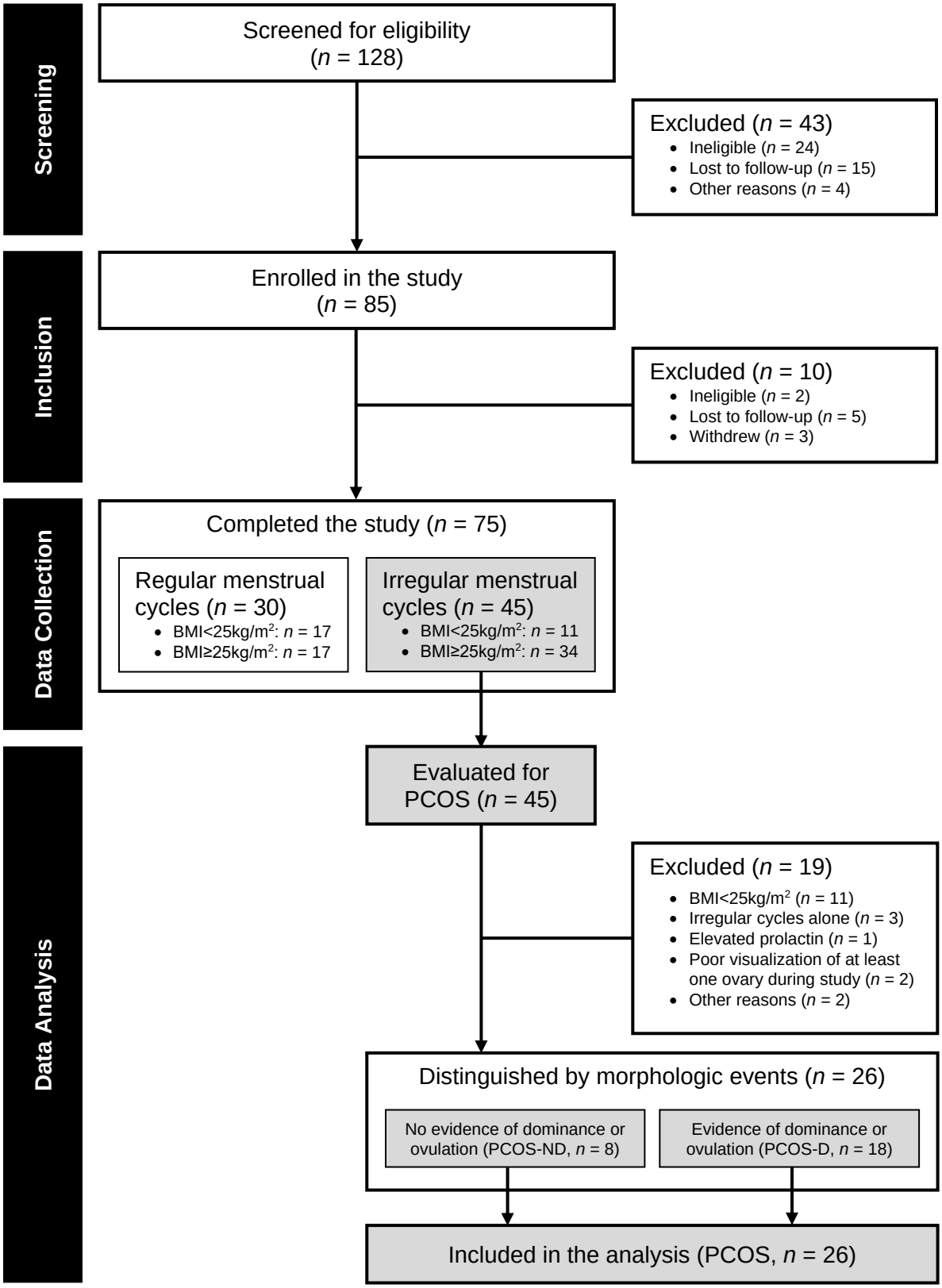
Ethical Considerations

This observational study included data that were collected as part of two study protocols (ClinicalTrials.gov Identifiers: NCT01927432, NCT01785719) between 2009 and 2016. Both protocols were approved by the Institutional Review Board at Cornell University, and informed consent was obtained from all participants before study procedures were initiated.

Study Participants

Twenty-six overweight and obese women (body mass index, BMI ≥25 kg/m^2) with irregular menstrual cycles and PCOS were recruited from the general population (Chapter 1 and Figure 3.1). PCOS was diagnosed by the Rotterdam criteria, as previously described (Chapter 1). To be eligible, participants had to be 18 to 38 years old, have normal serum concentrations of thyroid-stimulating hormone and prolactin,[7] and have consistent resolution of both ovaries on ultrasonography. Exclusion criteria included use of medications known or suspected to interfere with reproductive function in the two months prior to the study, recent pregnancy or lactation, history of premature ovarian failure or surgery, and medical conditions expected to interfere with study participation (Chapter 1).

Figure 3.1. Flow of participants through the observational cohort study (2009–2016) and present analysis. *Shaded boxes designate the cohorts from which participants were selected for the present analysis. Abbreviations: PCOS, polycystic ovary syndrome; BMI, body mass index.*



Ultrasonographic Measurements

Transvaginal ultrasonography was used to prospectively evaluate ovarian morphology (Chapter 1). Scans began at a random time and were performed every other day for 3–6 weeks. Follicular growth was closely monitored in real-time. If a large follicle (≥14 mm) was detected, then ultrasound examinations were performed daily until its regression or ovulation. Ovulation was identified by the observation of a corpus luteum.[73,74] Whole ovaries were imaged from their inner to outer margins in the longitudinal plane using a GE Voluson E8 Expert System and 6–12 MHz transducer (Chapter 1). Digital ultrasound images were archived for offline analysis by a single investigator (Santesoft LTD, Athens, Greece).

Ultrasound images of each ovary were analyzed for three parameters: (1) FNPO, (2) FNPS, and (3) OV. Cineloops throughout each ovary were evaluated for FNPO. Follicles ≥2 mm were counted and measured,[75] and the presence of a dominant follicle (≥10 mm) or corpus luteum was recorded. The largest cross-sectional view of each ovary was evaluated for FNPS and OV.[93] OV was calculated with the equation: π/6 × (transverse diameter) × (longitudinal diameter) × (anteroposterior diameter).[20,94] The image quality of each dataset was also subjectively categorized into one of three groups (1 = Poor, 2 = Partially Visible, 3 = Excellent), based on the proportion of the ovarian contour and follicles that could be seen. A value for FNPO, FNPS, OV, and image quality was designated as the mean recorded values of the left and right ovaries for each participant and day of the study.

Definitions

Following offline image analysis, participants were stratified into two groups based on the absence (PCOS-ND) or presence (PCOS-D) of a dominant follicle during the study (Figure 3.1). Data were then binned by cycle phase. *Early follicular phase* referred to a time after menses with no follicular development >9 mm. *Mid- to late-follicular phase* referred to a time from the first day a dominant follicle was detected to the last day before it ovulated, regressed, or the study ended.

Luteal phase referred to a time from the first day a corpus luteum was detected to the last day before menses or the study ended.

Statistical Analyses

Statistical analyses were performed with JMP Pro 12.0.1. (SAS Institute, Cary, NC); the threshold for statistical significance was set at $P \leq 0.05$. Descriptive statistics were tabulated for each endpoint. Normality was evaluated with histograms or residual plots, and skewed data were log-transformed prior to analyses. Cross-sectional data related to participant characteristics were compared between the PCOS-ND and PCOS-D groups using two-sample t-tests. Serial data related to sonographic endpoints were centralized to the first day of the study (PCOS-ND) or cycle phase (PCOS-D) and normalized over time. Group-specific mixed-effects models were performed to evaluate changes in FNPO, FNPS, and OV (i.e. an effect of day or cycle phase). The degree of intra-cycle versus inter-individual variation was considered. *Participant number*, *participant number* crossed with *time*, and *participant number* crossed with *image quality* were included as random effects in all models. Mean FNPO, FNPS, and OV across the anovulatory interval or cycle phase were calculated and a diagnosis was applied. The Cochrane Armitage Trend test was then used to determine differences in the probability of diagnosis over time.

RESULTS

Characteristics of Study Participants

Participant demographics and diagnostic features are presented in Table 3.1. Overall, women began the study at a random time, between four days and one year after their self-reported last menses. They were evaluated for an average of 31 days (range: 23–46 days). Eight of the 26 women with PCOS did not exhibit a morphologic event during the study (i.e. PCOS-ND). No dominant or ovulatory follicles or corpora lutea were observed. Therefore, data collected from the women in this group represented a random time during the early follicular phase. By contrast, 18 of the 26 women with PCOS exhibited at least one morphologic event during the study (i.e. PCOS-D). Dominance (n=18) and sporadic ovulation (n=13) were observed. Therefore, data collected from the women in this group represented three phases of the menstrual cycle: (1) the early follicular phase (n=18); (2) the mid- to late-follicular phase (n=18); and (3) the luteal phase (n=13). Women were observed for an average of 13 days in the first phase (range: 2–28 days), 7 days in the second phase (range: 2–33 days), and 7 days in the third phase (range: 1–16 days).

Demographics and diagnostic features were compared between the PCOS-ND and PCOS-D groups (Table 3.1). Despite meeting the Rotterdam definition of PCOS, women with PCOS-D had milder reproductive features than women with PCOS-ND. Namely, women with PCOS-D reported shorter menstrual cycles (P=0.04), albeit still above the threshold for oligo- or amenorrhea (i.e. >35 days), and had lower total testosterone concentrations (P=0.05) (Table 3.1).

Table 3.1. Characteristics of study participants

	PCOS (*n* = 26)	PCOS-ND (*n* = 8)	PCOS-D (*n* = 18)
Age (y)	27 ± 5 (20, 36)	26 ± 4 (21, 31)[a]	28 ± 5 (20, 36)[a]
Body mass index (kg/m^2)	35.3 ± 6.7 (25.7, 48.4)	36.8 ± 8.9 (26.4, 48.4)[a]	34.7 ± 5.7 (25.7, 46.2)[a]
Menstrual cycle length (d)	119 ± 106 (41, 365)[‡]	198 ± 144 (50, 365)[a‡]	78 ± 43 (41, 180)[b‡]
Hirsutism score	7 ± 4 (1, 15)	7 ± 4 (1, 14)[a]	7 ± 4 (2, 15)[a]
Total testosterone (ng/dl)	54.7 ± 25.9 (15.8, 118.0)	73.1 ± 31.4 (32.9, 118.0)[a]	46.0 ± 17.9 (15.8, 81.5)[b]
Free androgen index	8 ± 5 (1, 23)	11 ± 7 (4, 23)[a]	6 ± 4 (1, 15)[a]
Mean FNPO	42 ± 20 (14, 84)	54 ± 24 (14, 80)[a]	37 ± 16 (14, 84)[a]
Mean FNPS	8 ± 4 (2, 21)	9 ± 5 (3, 15)[a]	8 ± 4 (2, 21)[a]
Mean OV (ml)	14 ± 5 (7, 21)	17 ± 5 (7, 21)[a]	13 ± 4 (7, 20)[a]

Data are presented as mean ± SD (minimum, maximum). [a, b] Within rows, uncommon superscripts denote significant differences between the PCOS-ND and PCOS-D groups determined by t-test, $P \leq 0.05$. Diagnostic endpoints were evaluated on a single day of the scanning interval and with respect to stage of cycle. [‡] Data related to menstrual cycle length were censored to the year prior to enrollment (i.e. 365 days). *Abbreviations: PCOS, polycystic ovary syndrome; PCOS-ND, women without evidence of a dominant follicle during the study; PCOS-D, women with evidence of a dominant follicle during the study; FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*

Changes in Sonographic Markers of PCOM in Women with PCOS-ND

Mean profiles of FNPO, FNPS, and OV across the random anovulatory interval are illustrated for women with PCOS-ND in Figure 3.2. Little fluctuation was observed in any of the three sonographic markers. Mean values for FNPO and OV seemed constant over time and exceeded the current diagnostic thresholds for PCOM on each day of the study. Conversely, mean values for FNPS seemed slightly more variable over time and exceeded the diagnostic threshold on some, but not all, of the days (Figure 3.2). Mixed-effects model analysis confirmed these observations (Table 3.2). There was no effect of day for FNPO (P=0.23), FNPS (P=0.95), or OV (P=0.60). Intra-cycle fluctuation accounted for less than one-third of the total variance in each marker (FNPO: 3%; FNPS: 29%; OV: 14%). Most of the remaining variance was attributed to inter-individual factors (FNPO: 87%; FNPS: 41%; OV: 85%) or image quality. Image quality had the greatest impact on FNPS (30%) and the smallest impact on OV (1%) (Table 3.2).

Figure 3.2. Profiles (mean ± SD) of sonographic markers of polycystic ovarian morphology during one random scanning interval in women with PCOS-ND (*n* = 8). *The updated diagnostic threshold for each marker is depicted by a red line. Abbreviations: PCOS-ND, women without evidence of a dominant follicle during the study; FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*

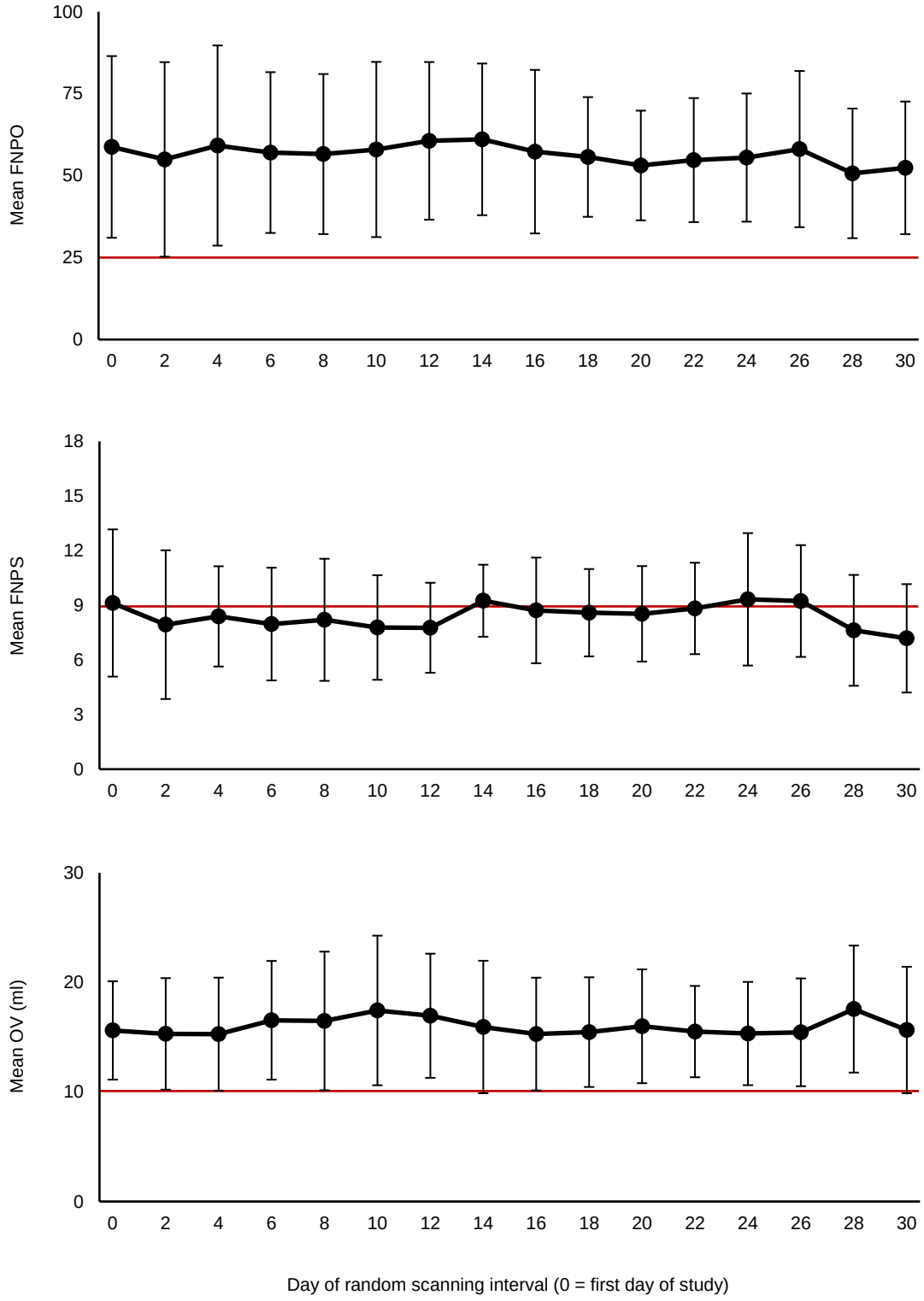


Table 3.2. Mixed-effects model analysis of changes in sonographic markers of polycystic ovarian morphology during a random scanning interval in women with PCOS-ND (*n* = 8)

	Intercept	Change over time		Components of total variance		
	β ± SE (95% CI)	β ± SE (95% CI)	*P* value	Intraindividual factors	Interindividual factors	Image quality
Mean FNPO	59 ± 8 (40, 77)	–0.2 ± 0.1 (–0.5, 0.1)	0.23	3%	87%	10%
Mean FNPS	9 ± 1 (7, 11)	0.0 ± 0.4 (–0.1, 0.1)	0.95	29%	41%	30%
Mean OV (ml)	16 ± 2 (11, 21)	0.0 ± 0.4 (–0.1, 0.1)	0.60	14%	85%	1%

Models included data collected every other day of the scanning interval (*n* = 16 data points per marker per woman). *Abbreviations: PCOS-ND, women without evidence of a dominant follicle during the study; FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*

Changes in Sonographic Markers of PCOM in Women with PCOS-D

Mean profiles of FNPO, FNPS, and OV across the different phases of the menstrual cycle are illustrated for women with PCOS-D in Figure 3.3. Fluctuation was apparent in two of the three sonographic markers. Whereas mean values for FNPO seemed constant over time, FNPS and OV seemed to change, particularly in the luteal phase. Similar to the random anovulatory interval, mean values for FNPO and OV exceeded the current diagnostic thresholds throughout each cycle phase. However, FNPS remained below the threshold on all days of the study (Figure 3.3). Mixed-effects model analysis confirmed these observations (Table 3.3). There was no effect of cycle phase for FNPO (P=0.48). However, FNPS (P<0.01) and OV (P<0.01) changed across the three cycle phases. Compared to the early follicular phase, the presence of a dominant follicle was associated with reduced values for FNPS (P=0.03). An active corpus luteum was also associated with reduced values for FNPS (P<0.01) and increased values for OV (P<0.02). Most of the variance was attributed to intra-cycle (FNPO: 14%; FNPS: 29%; OV: 33%) or inter-individual factors (FNPO: 82%; FNPS: 66%; OV: 66%). The impact of image quality was minimal (FNPO: 4%; FNPS: 4%; OV: 0%) (Table 3.3).

Figure 3.3. Profiles (mean ± SD) of sonographic markers of polycystic ovarian morphology across different phases of the menstrual cycle in women with PCOS-D*. The updated diagnostic threshold for each marker is depicted by a red line. Abbreviations: PCOS-D, women with evidence of a dominant follicle during the study; FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*

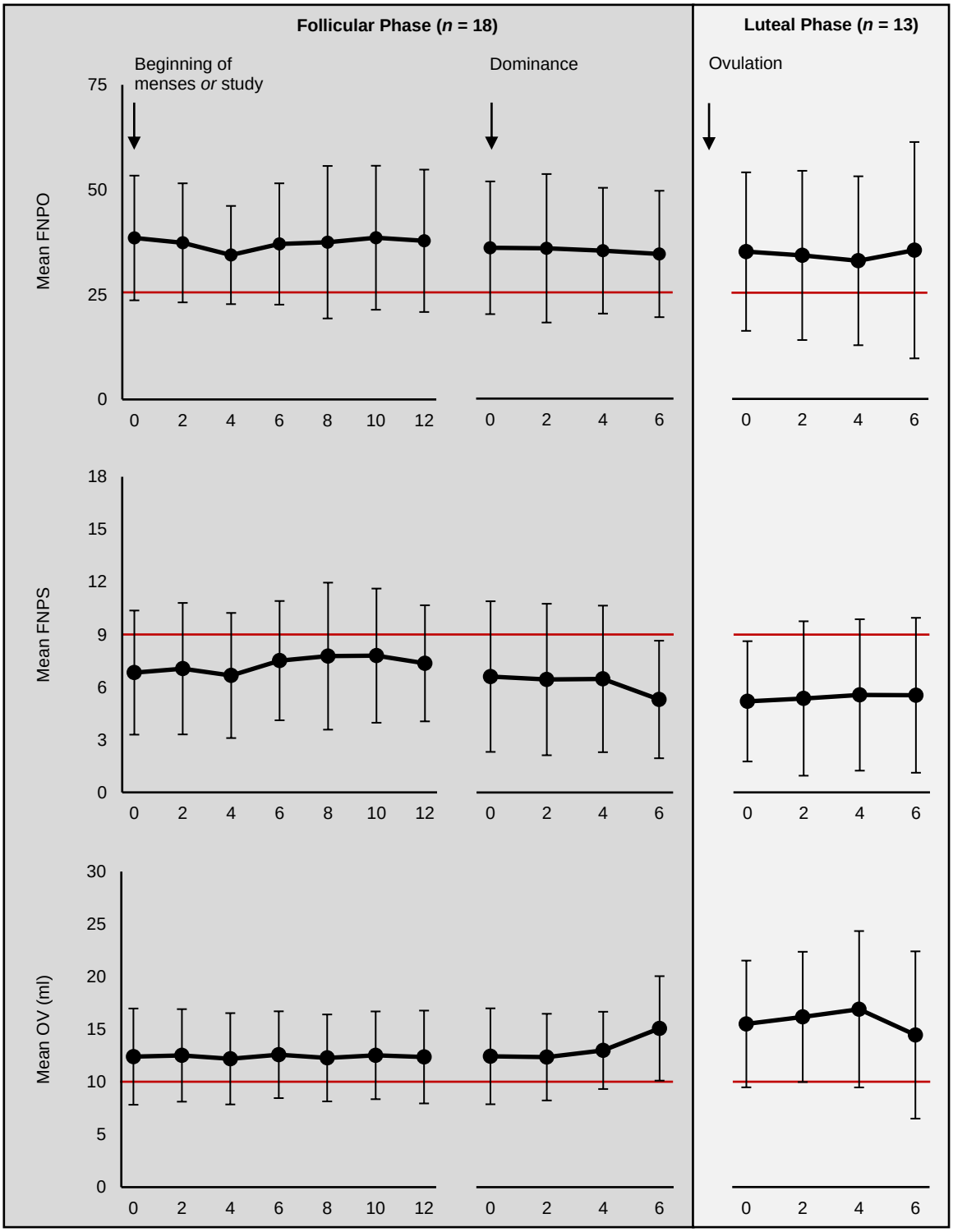


Table 3.3. Mixed-effects model analysis of changes in sonographic markers of polycystic ovarian morphology between different phases of the menstrual cycle in women with PCOS-D

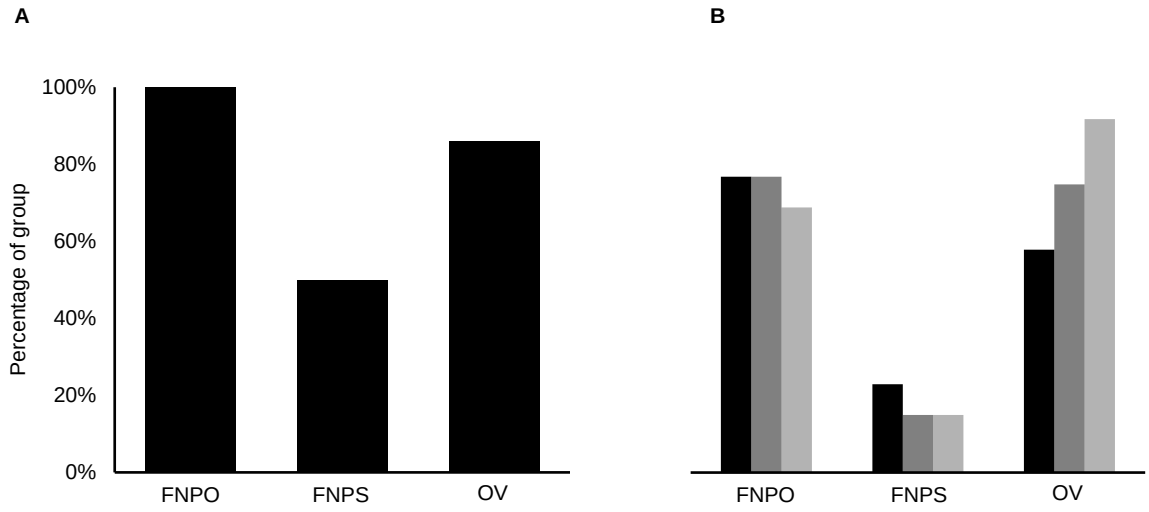
	Estimate per cycle phase			Overall change
	Early follicular phase (*n* = 18)	Mid- to late- follicular phase (*n* = 18)	Luteal phase (*n* = 13)	*P* value
Mean FNPO	38 ± 4 (29, 46)	36 ± 4 (27, 44)	35 ± 4 (27, 44)	0.48
Mean FNPS	7 ± 1 (6, 9)[a]	7 ± 1 (5, 8)[b]	6 ± 1 (4, 7)[b]	<0.01
Mean OV (ml)	12 ± 1 (10, 15)[a]	13 ± 1 (11, 16)[a]	16 ± 1 (13, 18)[b]	<0.01

Estimates per cycle phase are presented as least squares mean ± SE (95% CI). Models included data collected every other day during each cycle phase (*n* = 4–7 data points per marker per woman). [a, b] Within rows, uncommon superscripts denote significant differences between cycle phases determined by post-hoc Tukey's HSD tests, $p<0.05$. *Abbreviations: PCOS-D, women with evidence of a dominant follicle during the study; FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*

Proportion of Women Diagnosed with PCOM by Each Marker

The percentage of women diagnosed with PCOM by each marker is shown for the two groups in Figure 3.4. In the PCOS-ND group (*n*=8), most women met the current sonographic criteria for PCOM by FNPO (100%) and OV (86%). Only half of the women met the criteria by FNPS (50%). A similar case was apparent in the PCOS-D group (*n*=13). Across cycle phases, there were no differences in the percentage of women diagnosed with PCOM by FNPO (*P*=0.65) or FNPS (*P*=0.61). However, slightly more women were identified as having PCOM by OV in the luteal phase than in the follicular phase (*P*=0.06) (Figure 3.4).

Figure 3.4. Percentage of women meeting the updated diagnostic criteria for polycystic ovarian morphology (A) at any given time during the random scanning interval (*n* = 8) and (B) across different phases of the menstrual cycle (*n* = 13). *Mean data related to the early follicular phase (black), mid- to late-follicular phase (dark gray), and luteal phase (light gray) are represented. Abbreviations: FNPO, follicle number per ovary; FNPS, follicle number per cross-section; OV, ovarian volume.*



DISCUSSION

The objective of the present study was to evaluate the impact of a dominant follicle or corpus luteum on the morphologic diagnosis of PCOS. We observed that FNPO was constant across a random anovulatory interval (PCOS-ND) and different phases of the menstrual cycle (PCOS-D). We documented an increase in OV during the luteal phase, which contributed to an increase in the number of false-positive diagnoses of PCOM. Further, we found that FNPS was an inconsistent marker of PCOM over time and that its assessment was impacted by both image quality (PCOS-ND) and stage of cycle (PCOS-D).

Our results suggest that FNPO is a robust marker of PCOM in women with PCOS. FNPO consistently exceeded the current diagnostic threshold in women with (PCOS-D) and without (PCOS-ND) dominant follicle development. In addition, there was non-significant intra-cycle variation in FNPO across the random anovulatory interval (3%) and different phases of the menstrual cycle (14%). Minimal variation in FNPO during a random anovulatory interval is consistent with findings from our recent serial evaluation of antral follicle populations in PCOS (Chapter 1). The repeated findings are not surprising, because the same participants were included in both studies. However, the permanence of follicular excess over time is compelling. It reaffirms the predictive power of FNPO for the condition of PCOS[66,93] and speaks to the interchangeable nature of FNPO and antral follicle count (i.e. AFC; FNPO × 2) – a marker commonly used to assess ovarian reserve and response to assisted reproduction therapies.[109]

Greater variation in FNPO during the menstrual cycle versus anovulatory interval may be related to changes in the number of large follicles (≥5 mm), which are known to accompany follicular development in healthy women.[44] Indeed, significant changes in AFC (2–10 mm) have been reported during the mid-follicular and luteal phases in this population.[79] Although similar patterns were not detected in our study, there may be relevance to exploring alternative metrics to detect PCOM outside of the early follicular phase. Deb and colleagues noted that the number of small follicles (2–6 mm) showed little intra-cycle variation and excellent correlation between

different cycle phases in healthy women.[79] Similarly, we (Chapter 1) and others[57,103] have found that the number of small follicles (2–5 mm) can reflect follicular excess over time in women with PCOS. The evaluation of this follicle population in clinical practice is onerous, since counts are performed manually, and polycystic ovaries can contain >75 small follicles on any given day (Chapter 1). However, emerging ultrasound technology, designed to automatically count, measure, and classify follicles by size, holds promise to improve the efficiency of this process.[110] Future studies are needed to evaluate the utility of different metrics and automated technologies to assess PCOM.

Nevertheless, the clinical usefulness of AFC remains unchanged across the menstrual cycle in healthy women.[111,112] Variations in AFC do not impact its ability to reflect ovarian reserve or inform ovarian response to assisted reproduction therapies.[111,112] Similarly, the variations in FNPO did not impact its ability to detect PCOS over time in this study. We noted that inter-individual differences accounted for >80% of the variation in FNPO. Because the AFC is considered an accurate marker of the ovarian reserve,[109] this finding likely reflects differences in ovarian reserve among women in our cohort. It has been suggested that women with PCOS are born with a larger pool of resting follicles than controls,[113] but the extent to which ovarian reserve may differ among patients is unknown.

Our results suggest that FNPS is the least consistent marker of PCOM in women with PCOS. In both the PCOS-ND and PCOS-D groups, FNPS fell at or below the recommended diagnostic threshold on most days of the study. There was similar intra-cycle variation in FNPS across a random anovulatory interval (29%) and different phases of the menstrual cycle (29%). Such variation did not improve the ability of FNPS to detect PCOS over time. However, it indicated that assessments of the marker may be influenced by biological and technical factors. Specifically, substantial intra-cycle variation in FNPS reflected aspects of antral follicle development. Despite similar mean values over time, FNPS was significantly higher in the early follicular phase than in

the mid-to-late follicular or luteal phases. This suggests that FNPS was impacted by the presence of a dominant follicle and corpus luteum.

FNPS is widely used in clinical practice due to the convenience of acquiring still images versus video clips of the ovary. However, its utility compared with FNPO or OV is debatable.[19,66] We recently showed that FNPS has the poorest diagnostic potential of the three markers to detect PCOS.[93] We have hypothesized that this relates to the limitations of performing follicle counts when only a single cross-sectional view of the ovary is made available.[66] This approach relies heavily on the interpretive skills of the observer. It can be challenging to subjectively identify the largest plane of the ovary, resolve any anechoic regions, and discriminate between artifact and follicles under these conditions. We have previously reported that diagnostic confidence (as judged by the observer) in identifying PCOM is substantially lower when individuals are limited to performing follicle counts in a single cross-section compared to the entire ovary.[66] As a result, random fluctuations over time in FNPS might be expected. The notion that such fluctuation is linked to the observer is supported by our observation that image quality had a greater impact on FNPS than on other markers. We attempted to control for an impact of image quality by limiting study enrollment to women with excellent resolution of their ovaries on ultrasound. However, it was not unusual for an ovary to have reduced visibility from shadowing by bowel or the uterus on any given day. We suspect that we were more conservative with follicle counts in these cases, thereby resulting in lower detection rates of PCOM.

Our results suggest that OV is a robust marker of PCOM in women with PCOS – but only during the follicular phase. In both the PCOS-ND and PCOS-D groups, OV exceeded the current diagnostic threshold on each day of the study. There were substantial intra-cycle variations in FNPO across the random anovulatory interval (14%) and different phases of the menstrual cycle (33%). Intra-cycle variation during the random anovulatory interval was non-significant, but also greater than expected. Ovarian size has been shown to increase with the growth of a dominant follicle or corpus luteum.[108] However, these events did not occur during the anovulatory interval.

The absence of concurrent fluctuation in FNPO suggests that two-dimensional assessments of OV may suffer from the same technical limitations as described for FNPS. That is, selection of the largest cross-sectional view of the ovary may differ from day to day, and in the current analysis, may have impacted estimates of OV. Intra-cycle variation did not appear to change the likelihood of diagnosis with PCOM, but these outcomes could not be quantitatively assessed. Three-dimensional approaches may hold promise for more reliable evaluations of OV over time.[114]

Conversely, intra-cycle variation across different phases of the menstrual cycle was significant and expected.[108] OV increased from the follicular phase to the luteal phase. This observation reflected the growth of a corpus luteum in the luteal phase, which has been shown to comprise a large proportion of the ovarian tissue.[74] Substantial inter-individual variation (66%) in OV was likely related to differences in the types and sizes of corpora lutea detected among women.[74] An increase in OV during the luteal phase contributed to several false-positive diagnoses of PCOM. When evaluated by this marker alone, four participants transitioned from normal ovarian morphology in the follicular phase (i.e. OV <10 ml) to PCOM in the luteal phase (i.e. OV >10 ml). Notably, each of these women consistently met the diagnostic criteria for PCOM by FNPO during the menstrual cycle. This is in line with our previous observations that FNPO has greater diagnostic potential compared with OV.[66,93] Consequently, the use of FNPO to detect PCOM in these women may have obviated any concerns over misdiagnosis. Evaluation of OV in the non-dominant ovary (rather than taking the mean of both ovaries) may also be an appropriate approach in these cases. Clinicians often rely on metrics taken in the largest ovary to assign a diagnosis of PCOM. However, very little is known about inter-individual differences in ovarian morphology. Data from our group[115] and others[116] have suggested that the right ovary is larger than the left in controls and women with PCOS. Future studies are needed to corroborate an impact of sided differences on the diagnosis of PCOM before a recommendation can be made.

Last, we noted that image quality had the smallest impact on OV across the random anovulatory interval (1%) and different phases of the menstrual cycle (0%). This is consistent with

the current recommendation that OV be used to detect PCOM in the event of poor image quality or with older imaging technology (i.e. <8 MHz).

It is important to acknowledge that the actual relevance of ovarian morphology to the diagnosis of PCOS is unknown. Currently, the confirmation of PCOM on ultrasonography does not modify a diagnosis based on the presence of oligo- or anovulation and hyperandrogenism.[8,9,17] We found that 69% of the women in our study had a hyperandrogenic phenotype (i.e. Frank or Non-PCOM PCOS). Ultrasonography may only be helpful for these patients when access to a reliable androgen assay is limited.[117] Conversely, 31% of the women in our study had a normoandrogenic anovulatory phenotype (i.e. Mild PCOS). Reliable metrics to detect PCOM are the most relevant for this population. On average, women in the PCOS-D group were normoandrogenic and appeared to have lower FNPO and OV, though the differences did not reach significance. Future studies are needed to resolve whether the revised criteria for PCOM can adequately distinguish normoandrogenic women from healthy controls across the menstrual cycle. It is possible that the addition of functional ovarian markers (i.e. anti-Müllerian hormone) may improve the sonographic evaluation of PCOS.[118]

A major limitation of this study was the evaluation of only overweight and obese women with PCOS. The current morphologic criteria for PCOS were established in a cohort of >1,000 healthy women with normal BMIs. This is problematic, because overweight and obese women show a tendency for larger ovaries compared to their normal-weight counterparts, irrespective of PCOS status.[19] Emerging data from our group and others also suggest that obesity adversely impacts folliculogenesis in women with and without PCOS.[84,119,120] Future studies are needed to assess the impact of BMI on the morphologic criteria for this condition. As such, we acknowledge that the outcomes reported in this study cannot be readily extrapolated to normal-weight women. In addition, we recognize that sonographic evaluations of PCOM are usually conducted after a progestin-induced withdrawal bleed in clinical practice. Our assessments occurred during a natural cycle after an extended period of anovulation. To the best of our knowledge, there are

little to no data assessing the dynamics of luteal function in women with PCOS. Therefore, it is unclear whether aspects of PCOM differ during a pharmacologically-induced versus natural follicular phase.

In summary, FNPO showed less intra-cycle fluctuation than OV and FNPS in women with PCOS. This suggests that FNPO is a robust marker of PCOM and that it can be assessed at a random time regardless of cycle phase. In the event of poor image quality or older imaging technology, OV can be used as an alternative marker of PCOM. However, caution should be taken during the luteal phase to minimize the risk of misdiagnosis. The predictive power of follicle counts and ovarian size for PCOS have been described elsewhere.[66,93] This work extends these observations to further support FNPO as the most convenient marker of PCOM and strengthens the recommendation against the use of FNPS on its own to predict PCOS.

CHAPTER 4

IMPACT OF HYPOCALORIC DIETARY INTERVENTION ON OVULATION IN OBESE WOMEN WITH PCOS

Jarrett BY, Lujan ME. Impact of hypocaloric dietary intervention on ovulation in obese women with PCOS. Reproduction 2017; 153; R15–R27.

ABSTRACT

Polycystic ovary syndrome (PCOS) is a common cause of ovulatory dysfunction impacting women of reproductive age. Obesity and insulin resistance are thought to potentiate disruptions in antral follicle development that result in chronic anovulation, and as such, have become important therapeutic targets of dietary interventions aimed at weight loss. Caloric restriction has been shown to promote sporadic ovulation in obese women with PCOS, but improvements have occurred across a wide range of patients and little has been garnered about the factors that distinguish responders from non-responders. Further, few studies have evaluated the likelihood for modest weight loss to restore normal ovulatory cyclicity in PCOS. Consensus regarding the impact of dietary intervention on ovulation has been limited by variability in the measures used to characterize and report ovulatory status across studies. In response, this review provides an assessment of the evidence surrounding the effectiveness of hypocaloric dietary intervention to normalize ovulatory function in PCOS. The impact of physiological versus methodological factors on the evaluation of ovulatory status is discussed and recommendations to strengthen future studies in this area are provided. Ultimately, further research is needed to understand the optimal dietary or lifestyle approaches that promote ovulation and sustained improvements in reproductive function in PCOS.

INTRODUCTION

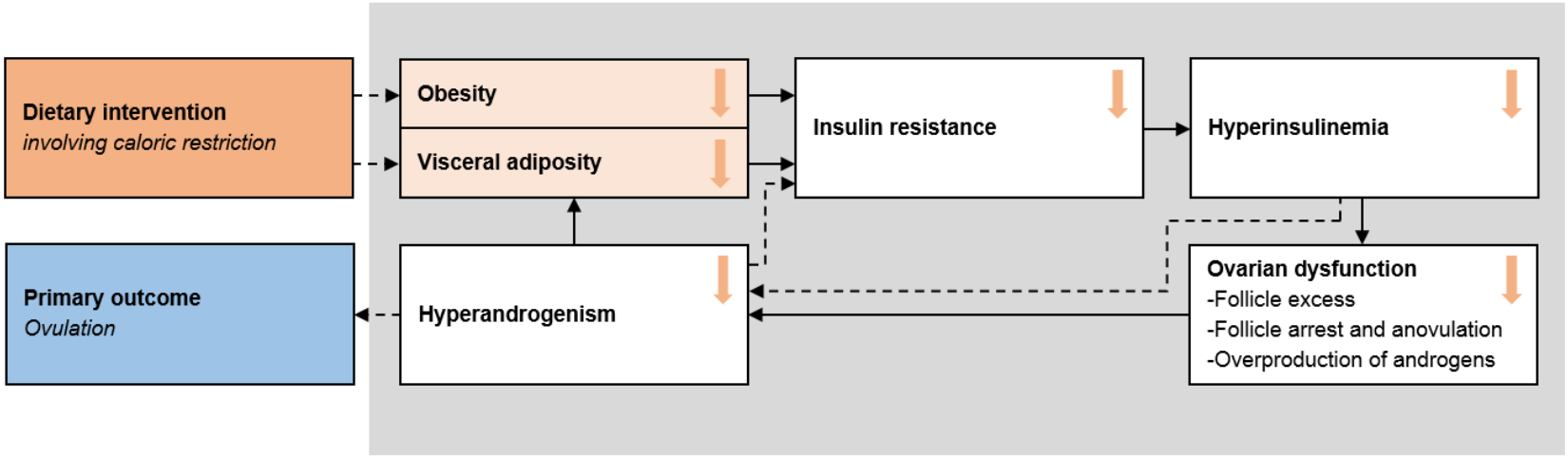
Polycystic ovary syndrome (PCOS) is the leading cause of anovulatory infertility and has broad implications for the reproductive and metabolic health of women across the lifespan.[2] The reproductive phenotype manifests as anovulation, menstrual irregularity, and hyperandrogenism and reflects defects at multiple levels of the hypothalamic-pituitary-ovarian axis.[47,46] The hypersecretion of luteinizing hormone by the pituitary and overproduction of androgens by the ovaries interact to impair ovarian antral follicle development.[47,46] Abnormal folliculogenesis in PCOS is characterized by an accumulation of small follicles, inhibition of terminal follicular growth (called follicle “arrest”), and failure of the mechanisms driving morphologic selection and ovulation.[47,46] Obesity is intimately linked with the pathogenesis of anovulation in PCOS (Figure 4.1).[67] Excess weight and visceral adiposity promote the development of insulin resistance and compensatory hyperinsulinemia,[89] which are posited to exacerbate disruptions in antral follicle development[47,46] and worsen the severity of the reproductive phenotype.[68] Therefore, therapies that attenuate obesity and insulin resistance hold promise to normalize anovulation and hyperandrogenism in PCOS (Figure 4.1).

Currently, dietary interventions involving caloric restriction are recommended to combat both reproductive and metabolic abnormalities in overweight and obese women with PCOS.[2,7,62,121] Modest reductions in energy intake (500–1000 kcal/d) and weight (5–10%) have been shown to normalize gonadotropin secretion,[122] reduce clinical and biochemical hyperandrogenism and improve insulin sensitivity in this population.[62,123] Likewise, randomized and non-randomized trials have documented an increased frequency of spontaneous ovulation, menses and pregnancy with weight loss.[62] These findings are thought to reflect a recovery of the hormonal features that underpin follicular excess and “arrest” in PCOS (Figure 4.1).[123] However, there are several challenges to understanding the actual effectiveness of weight loss to stimulate ovulation or restore normal ovulatory function in overweight and obese patients. Despite evidence of ovulation following dietary interventions, variability in the measures used to report endpoints

across studies has prevented systematic assessments of the impact of weight loss on reproductive outcomes.[123] Further, improvements in ovulation have been noted over a wide range of women,[7,62] yet little work has been done to understand the factors that account for variability in the ovulatory response to caloric restriction.

To that end, the purpose of this review was to assess the evidence surrounding the effectiveness of hypocaloric dietary intervention to normalize ovulatory function in PCOS. The occurrence of and factors associated with ovulation in response to modest weight loss are described. Particular consideration is given to inconsistencies in the methods used to characterize ovulatory status across studies. The impact of physiological and methodological factors on the evaluation of ovulatory potential prior to dietary intervention is also discussed.

Figure 4.1. Proposed mechanism by which hypocaloric dietary intervention promotes ovulation in obese women with PCOS. *As shown in the gray inset, excess weight and visceral adiposity promote the development of insulin resistance and compensatory hyperinsulinemia. Both are posited exacerbate disruptions in antral follicle development, through a reciprocal relationship with hyperandrogenism. Insulin contributes to systemic hyperandrogenism by stimulating the production of androgens by the ovaries and inhibiting the synthesis of sex hormone-binding globulin by the liver. These direct (solid line) and indirect actions (dotted line) of insulin result in elevated circulating concentrations of bioavailable androgens. Androgens may promote preferential deposition of adipose tissue in the abdomen and can exacerbate insulin resistance through obesogenic mechanisms (solid line) or independently (solid line) by modifying insulin action in classic target tissues. Since dietary interventions attenuate obesity and insulin resistance (orange boxes and arrows), they hold promise to treat anovulation and hyperandrogenism in PCOS.*



LITERATURE SEARCH AND SELECTION CRITERIA

PubMed, CINAHL and the Cochrane Central Register of Controlled Trials were used to identify relevant studies published between January 1990 and March 2016. Bibliographies of related systematic or narrative review articles were also screened to identify additional studies. The search included a combination of keywords relevant to PCOS, dietary or lifestyle intervention, and ovulation and menstrual cyclicity. The Population, Intervention, Comparison, Outcome (PICO) framework was used to define the inclusion and exclusion criteria for studies *a priori*. Briefly, studies included in this review were limited to original research articles in which: (1) the patient population comprised only overweight or obese women with PCOS; (2) the dietary intervention involved reductions in energy intake that were intended to promote weight loss; and (3) the primary or secondary outcome of interest was ovulation. Only articles published in English were included. Overweight or obesity was defined as a BMI of 25.0–29.9 kg/m^2 (overweight) or ≥30 kg/m^2 (obesity).[124] PCOS was defined according to the National Institutes of Health (NIH)[17] or Rotterdam criteria.[8,9] Randomized controlled trials and non-randomized intervention studies were considered. Trials that incorporated either supervised or unsupervised physical activity with caloric restriction were included. By contrast, studies with combined dietary and pharmaceutical interventions (i.e. metformin or clomiphene citrate) were excluded, unless the pharmaceutical therapy served as a comparison to changes in energy intake alone. The title and abstract of every record retrieved by this search strategy was checked to ensure that it aligned with the established inclusion criteria. Relevant articles were downloaded for full-text review. Data on general characteristics of the study, patient population, diagnosis of PCOS, inclusion and exclusion criteria, intervention design, measurement of ovulation and outcomes related to ovulatory function were extracted.

RESULTS

Characteristics of the Studies Included for Review

The search returned 4,046 records, including ones that were identified through electronic databases (*n*=3,319) and bibliographies of other reviews (*n*=727). Duplicates found using multiple databases, keywords and sources were removed (*n*=3,234). All remaining records (*n*=812) were evaluated in the context of the PICO framework, and 780 were excluded based on the information provided in the title and/or abstract. Thirty-two original research articles were selected for full-text review. Of these, 13 were excluded due to the inclusion of women without PCOS (*n*=2), use of inappropriate diagnostic criteria (*n*=1), use of eucaloric dietary interventions (*n*=3), or absence of outcome data on ovulation (*n*=7). Ultimately, 19 articles from 17 different studies were included for review. Two of the articles constituted secondary analyses[125,126] of data collected during a previous dietary intervention.[127,128]

Relevant study characteristics are summarized in Table 4.1. At enrollment, the mean age of participants ranged from 23 to 33 years and mean BMI was within the obese category (Table 4.1, Column 2). All of the dietary interventions were aimed at modest weight loss, primarily through short-term reductions in energy intake. In general, women were prescribed a caloric deficit of at least 500 kcal per day and encouraged to restrict energy intake to between 1,000 and 1,400 kcal per day. Two studies used very low calorie diets (i.e. total energy intake <500 kcal per day)[129,130] (Table 4.1, Column 4). Despite being hypocaloric, the dietary interventions met national standards for carbohydrates (45–65% of calories), protein (10–35% of calories) and fat (20–35% of calories).[131] Participants were largely required to purchase and prepare their own meals and snacks; four studies provided partial or complete meal replacements to aid in caloric restriction.[127,129,130,132] Multifactorial approaches to enhance the target energy deficit and degree of weight loss were also common. Some studies modified macronutrient composition, recommended a specific frequency or intensity of physical activity, and/or delivered behavior

modification therapy through individual or group education sessions (Table 4.1, Column 5). Fifteen of the 17 studies were conducted over a period of three months or longer, wherein the duration of active weight loss ranged from six weeks[133] to seven months[134–137] (Table 4.1, Columns 3–4). At the end of the dietary interventions, mean reductions in body weight ranged from 3%[128,136] to 16%[138] (Table 4.1, Column 6).

Improvements in ovulation were defined in one of two ways across studies: (1) the occurrence of one or two ("sporadic") ovulations or (2) the resumption of regular ("monthly") ovulatory cycles. Data were reported as the proportion of women with either ovulatory response or as the number of ovulatory menstrual cycles detected during the dietary intervention. Overall, most of the results on improved ovulatory function were presented as the number of women who experienced sporadic ovulation with weight loss. Only two of the studies assessed the occurrence of regular ovulatory cycles[139,140] and none compared the likelihood for dietary intervention to stimulate a single ovulation versus restore normal ovulatory function. Similarly, three studies published data on the number of ovulatory menstrual cycles detected per woman or treatment group, but did not provide any additional information with which to characterize ovulatory cyclicity.[134,135,137]

Table 4.1. Summary of studies of the impact of hypocaloric dietary intervention on ovulation in overweight or obese women with polycystic ovary syndrome (PCOS)

First Author	Population and Diagnosis	Study Design	Caloric Restriction	Additional Approaches	Weight Loss
Guzick (1994)	n=6 Age 32y, IBW 176% OA + HA	3 months RCT (diet vs. waiting list)	2 months: 400 kcal/d 1 month: 1000–1200 kcal/d	Behavior modification PA goal of 2 mi/d, 5 d/wk	16.2 kg (15%) decrease in weight
Crosignani (2003)	n=27 Age 31y, BMI 32.1kg/m^2 OA + PCO	Variable (≤6 months) NRS (single cohort)	1200 kcal/d	PA goal of 1–2 d/wk	76% of subjects lost 5–10% body weight
Moran (2003)	n=28 Age 33y, BMI 37.4kg/m^2 OA + HA	4 months RCT (LP vs. HP; during both caloric restriction & WMD)	3 months: 1400 kcal/d 1 month: WMD	HC/LP or LC/HP diets Behavior modification Supervised PA for 60 min/wk, with goal of ≥2 other d/wk	Cohort: 8 kg (8%) decrease in weight Decrease in weight in LP (7 kg) vs. HP (9 kg), NS
Hoeger (2004)	n=6 Age 27y, BMI 40.0kg/m^2 OA + HA	12 months RCT (diet vs. no diet)	6 months: ≥500 kcal/d deficit 6 months: WMD	Low glycemic index foods Behavior modification PA goal of 150 min/wk	7 kg decrease in weight
van Dam (2004)	n=15 Age 29y, BMI 39.0kg/m^2 OA + HA	Variable (~7 months) NRS (single cohort)	470 kcal/d	N/A	≥10% decrease in weight
Moran (2006, 2007a)	n=33 Age 32y, BMI 34.9kg/m^2 Rotterdam; all phenotypes	8 months RCT (FC vs. CC; WMD)	2 months: 2 meal replacements/d; 1170 kcal/d 6 months: WMD (FC vs. CC)	Behavior modification PA goal of 8,000 steps/d	Cohort: 5.6 kg (9%) decrease in weight Decrease in weight in R (6%) vs. NR (7%), NS
Moran (2007b)	n=15 Age 32y, BMI 35.7kg/m^2 Rotterdam; all phenotypes	2 months NRS (PCOS vs. control)	2 meal replacements/d	N/A	3.9 kg (4%) decrease in weight
Qublan (2007)	n=21 with OA Age 32y, BMI 32.2kg/m^2 Rotterdam; all phenotypes	Variable (≤6 months) NRS (diet vs. metformin)	1200–1400 kcal/d	N/A	4.8 kg/m^2 (15%) decrease in BMI
Palomba (2008)	n=20 Age 26y, BMI 33.2kg/m^2 OA + HA + PCO	6 months NRS (diet vs. PA)	800 kcal/d deficit	LC/HP diet Behavior modification	Decrease in weight in R (11 kg) vs. NR (2 kg), $p<0.05$

Table 4.1, continued.

First Author	Population and Diagnosis	Study Design	Caloric Restriction	Additional Approaches	Weight Loss
Thomson (2008)	n=53 with OA Age 29y, BMI 36.1kg/m^2 Rotterdam; all phenotypes	5 months RCT (DO vs. DA vs. DC)	1200–1400 kcal/d	LC/HP diet Behavior modification Supervised PA for 5 d/wk	Cohort: 9% decrease in weight Decrease in DO (9%) vs. DA (10%) vs. DC (8%), NS
Thomson (2009)	n=52 Age 30y, BMI 36.5kg/m^2 Rotterdam; OA only	5 months NRS (single cohort)	1400 kcal/d	N/A	Cohort: 9 kg decrease in weight Decrease in weight in R (12 kg) vs. NR (6 kg), p<0.05
Palomba (2010)	n=32 Age 28y, BMI 31.3kg/m^2 OA + HA + PCO	1.5 months RCT (diet vs. clomiphene)	1000 kcal/d deficit	LC/HP diet Behavior modification Supervised PA for 3 d/wk	4 kg (5%) decrease in weight
Fux Otta (2010)	n=15 Age 25y, BMI 35.6kg/m^2 OA + HA	4 months RCT (diet vs. metformin)	1500 kcal/d	PA goal of ≥40 min/d, 4 d/wk	1.4 kg/m^2 (4%) decrease in BMI
Kuchenbecker (2011)	n=32 Age 25y, BMI 35.6kg/m^2 Rotterdam; OA only	6 months NRS (single cohort)	≥500 kcal/d deficit	Behavior modification Individualized PA goals	Decrease in weight in R (6%) vs. NR (3%), p<0.05
Ladson (2011)	n=16 Age 29y, BMI 38.3kg/m^2 OA + HA	6 months RCT (diet vs. metformin)	500 kcal/d deficit	HC/LP diet Supervised and unsupervised PA, with goal of 150 min/wk	N/A
Nybacka (2011, 2013)	n=43 Age ~31y, BMI ~36.1kg/m^2 OA + HA + PCO	4 months RCT (diet vs. PA vs. diet/PA)	≥600 kcal/d deficit	HC/LP diet Behavior modification Individualized PA goals	Decrease in weight in diet (6%) vs. PA (3%) vs. diet/PA (5%), NS
Pasquali (2011)	n=65 Age ~23y, BMI ~34.8kg/m^2 OA + HA	Variable (≥6 months) NRS (single cohort)	6 months: 1200–1400 kcal/d Variable follow-up period: WMD	PA goal of 30 min/d, 5 d/wk	Decrease in weight in R (16%) vs. NR (13%), NS

Values for clinical characteristics and weight loss are presented as means. Sample sizes refer to the number of women with anovulatory phenotypes at baseline who completed the hypocaloric dietary intervention. Where possible, changes in weight are reported for responders (R) versus non-responders (NR) and correspond to data on ovulation presented in Table 4.2. "Response" was broadly defined as any improvement in ovulatory or menstrual function. Across studies, behavior modification was defined as interactive individual or group education with a health care provider. *Abbreviations: OA, oligo-amenorrhea; HA, hyperandrogenism; PCO, polycystic ovaries; IBW, ideal body weight; BMI, body mass index; RCT, randomized controlled trial; NRS, non-randomized study; HC/LP, high-carbohydrate/low-protein diet; LC/HP, low-carbohydrate/high-protein diet; FC, fat-counting; CC, carbohydrate-counting; WMD, weight maintenance diet; PA, physical activity; DO, dietary intervention; DA, dietary intervention with aerobic exercise; DC, dietary intervention with combined aerobic and resistance exercise; NS, not significant at p<0.05; N/A, not reported.*

Dichotomization of Women as Responders and Non-Responders to Dietary Intervention

Based on these definitions, the ovulatory response to hypocaloric dietary intervention was decidedly variable between individuals and across studies (Table 4.2, Columns 6–7). Both sporadic and regular ovulations were detected in a wide range of participants with either self-reported or confirmed menstrual cycle irregularity at baseline. Thirteen to 85% of women experienced sporadic ovulation with weight loss and no more than 55% resumed regular ovulatory cycles (Table 4.2, Columns 6–7). On average, fewer than three ovulations were detected per woman during a six-month dietary intervention.[134,135,137] Together, these data imply continued cycle irregularity with ovulation occurring at least two months apart.

In all studies, a subset of women remained anovulatory despite being compliant with the dietary intervention (as judged by weight loss) (Table 4.2, Columns 6–7). Hence, women could be dichotomized as “responders” and “non-responders” to weight loss. These results could be interpreted to mean that dietary intervention is not a universal solution for anovulation in PCOS. Yet, it is prudent to consider that variability in the ovulatory response may stem from (a) inconsistencies in the measurement of ovulation across studies, (b) heterogeneity in the clinical presentation of PCOS or (c) the degree of change in salient endocrine or metabolic features during the dietary intervention. The potential impact of each of these factors in the evaluation of the ovulatory response to weight loss is addressed in the sections that follow.

Table 4.2. Differences in the measurement of ovulation and ovulatory response to hypocaloric dietary intervention across studies in overweight or obese women with PCOS

First Author	Measurement of Ovulation		Evaluation of Baseline Ovulatory Function	Length of Caloric Restriction	Proportion of Subjects with Evidence of Ovulation during Intervention	
	Marker	*Frequency*	*Marker (Duration)*		*Sporadic Ovulation*	*Regular Ovulation*
Guzick (1994)	Serum P4	Weekly, 8 weeks post-diet	Biochemical (2 months)[b]	3 months	4/6 (67%)	N/A
Crosignani (2003)	Serum P4	After self-report of regular menses	Not completed	Variable (≤6 months)	N/A	15/27 (55%)
Moran (2003)	Urinary PDG	Twice weekly	Menses diary (6 months)	3 months	22/28 (79%)	N/A
Hoeger (2004)[a]	Urinary PDG	Weekly	Not completed	6 months	N/A	N/A
van Dam (2004)	Serum P4	Biweekly	Biochemical (Once)[b]	Variable (~7 months)	9/15 (60%)	N/A
Moran *(*2006, 2007a)	Urinary PDG	Twice weekly	Menses diary (6 months)	2 months	28/33 (85%)	N/A
Moran (2007b)	Urinary PDG	Twice weekly	Menses diary (6 months)	2 months	11/15 (73%)	N/A
Qublan (2007)	Serum P4	After self-report of regular menses	Not completed	Variable (≤6 months)	5/21 (24%)	N/A
Palomba (2008)	Plasma P4	After spontaneous/induced menses	Not completed	6 months	5/20 (25%)	N/A
Thomson (2008)	Urinary PDG	Twice weekly	Biochemical (1 month)	5 months	N/A	12/53 (23%)
Thomson (2009)	Urinary PDG	Twice weekly	Biochemical (1 month)	5 months	10/52 (19%)	N/A
Palomba (2010)	Plasma P4	After visualization of CL on TVUS	Not completed	1.5 months	4/32 (13%)	N/A
Fux Otta (2010)	Serum P4	Uncertain	Menses diary (6 months)	4 months	6/15 (40%)	N/A
Kuchenbecker (2011)	Serum P4	After self-report of increase in BBT	Not completed	6 months	14/32 (44%)	N/A
Ladson (2011)[a]	Urinary PDG	Daily	Not completed	6 months	N/A	N/A
Nybacka (2011, 2013)	Serum P4	After self-report of menses	Not completed	4 months	15/43 (35%)	N/A
Pasquali (2011)	Serum P4	Once pre- and post-diet	Biochemical (Once)	Variable (6 months)	35/65 (54%)	N/A

Outcomes are reported for women with anovulatory phenotypes prior to dietary intervention. [a] Data were reported in an inconsistent format compared to other studies and could not be extracted. [b] Results of baseline assessments were reported in the article. *Abbreviations: P4, progesterone, PDG, pregnanediol glucuronide; CL, corpus luteum; TVUS, transvaginal ultrasonography; BBT, basal body temperature; N/A, not reported.*

Inconsistencies in the Methods Used to Measure Ovulatory Status

In line with previous reports,[123] the methods used to detect ovulation and characterize any reinstatement of ovulatory cyclicity were inconsistent across studies (Table 4.2, Columns 1–3). As shown in Table 4.2 (Column 2), the primary markers of ovulation were elevated serum concentrations of progesterone or increased urinary excretion of pregnanediol 3-glucuronide (PDG). These measurements were consistently performed during the mid-luteal phase of the menstrual cycle. Several studies also evaluated urinary estrogens in conjunction with PDG[141] or considered pregnancy to be sufficient evidence of ovulation in lieu of serum progesterone.[136,139]

In both clinical practice and research, a number of techniques are used to confirm the occurrence and timing of ovulation. The gold standard method involves direct observation of follicular growth and rupture by high-resolution transvaginal ultrasonography.[22] If this approach is not feasible, then other indirect, but objective, methods may be employed to detect ovulation. Such methods include the measurement of pituitary or ovarian hormones in the blood, urine or saliva, and the assessment of clinical symptoms including menstrual cycle length or basal body temperature (BBT).[142,143] In line with these standards, the studies were justified in their common use of serum progesterone or urinary PDG (Table 4.2, Column 2); both have been validated as reliable and interchangeable markers of ovulation in women with regular menstrual cycles.[144] A sustained elevation in either biochemical marker is considered sufficient evidence of luteal activity and can be detected throughout the luteal phase to the end of the cycle.[144]

That being said, the frequency at which these biochemical markers were measured differed substantially across studies. Approximately half of the studies (*n*=8) used an intermittent sampling design irrespective of the stage of the menstrual cycle. Assessments were performed daily,[137] twice weekly,[127,132,140,141,145] weekly,[134] or biweekly[130] to detect ovulation during the dietary intervention (Table 4.2, Column 3). By contrast, six studies collected a single blood sample in the mid-luteal phase based on morphologic or clinical markers.[128,133,135,136,139,146] Of these, only one used direct methods to monitor follicle growth and determine ovulation.[133] Namely, Palomba and

colleagues performed transvaginal ultrasonography at baseline, every four days until visualization of a dominant follicle and then daily to follicular collapse. Ovulation was subsequently confirmed with plasma progesterone levels.[133] The other five studies relied on participant self-report of recent spontaneous menses,[128,135] resumption of regular menses[139,146] or increase in BBT[136] (Table 4.2, Column 3). When one of these events occurred, the investigators scheduled a blood draw for serum progesterone based on an estimation of time to the next luteal phase.[128,135,136,139,146] Finally, of the remaining three studies, one did not provide adequate information with which to judge the frequency of measurements[147] and two did not assess ovulation until after the intervention had ended[129,138] (Table 4.2, Column 3).

It is probable that the studies with intermittent sampling designs were better positioned to capture ovulatory status than the studies that relied on a single biochemical measurement or self-reported clinical data. The occurrence of ovulation is best ascertained with an intermittent sampling design that allows for hormone data to be collected either daily, every-other-day, twice weekly, or weekly.[148] If one of these approaches is not feasible, a single measurement of serum progesterone or urinary PDG in the mid-luteal phase can also be effective to determine whether ovulation has occurred.[149] However, the reliability of a single measurement to detect ovulation during long or unpredictable menstrual cycles, capture peak progesterone concentrations or judge luteal phase sufficiency is uncertain. Similar to biochemical methods, prospective menstrual diaries and BBT records have been shown to provide reliable estimates of menstrual regularity[150] and the presence of ovulation in healthy women.[142,143] Yet, variability in consecutive cycle lengths is common[150] and ovulation is not an inevitable event during spontaneous menstrual cycles.[151] Infertile patients also demonstrate abnormal fluctuations in BBT during ovulatory cycles, leading to errors in the interpretation of temperature charts and prediction of ovulation.[152] Given these challenges with self-reported clinical data, the choice to schedule blood draws during the predicted luteal phase of the next menstrual cycle implies that the first ovulation was never biochemically confirmed.[128,135,136,139,146] Such approaches may have resulted in a poor estimation

of the number of ovulatory cycles, and consequently, a reduced likelihood to accurately report the ovulatory response to weight loss.

There was also marked variability in the methods used to define biochemical evidence of ovulation across studies. In the interventions that assessed serum progesterone (Table 4.2, Column 2), ovulatory thresholds were always reported and ranged from ≥4 ng/ml (13 nmol/L)[147] to ≥10 ng/ml (32 nmol/L).[133,135,146] It was difficult to discern whether these thresholds were internally validated or reflected the limits of detection of the various assays used. Two of the studies implemented a commercial radioimmunoassay,[130,146] but the others did not describe the methods employed to measure serum progesterone. By contrast, normative values for PDG were not provided by any of the studies that used the marker and the increase in mid-luteal excretion was qualitatively assessed.[127,128,132,134,140,141,145]

Accordingly, the interpretation of ovulatory status may have been limited by the use of inappropriate thresholds for progesterone and PDG to confirm ovulation. In general, a serum progesterone concentration of 3.9 ng/ml (12.5 nmol/L) or higher is thought to be presumptive evidence of an ovulatory cycle using commonly available commercial assays.[149,153] However, all of the studies identified thresholds for progesterone that were above this concentration.[129,130,133,135,136,138,139,146,147] This is interesting, considering that the studies that relied on the highest thresholds (≥32 nmol/L) were also the ones to report the smallest proportion of responders to the dietary intervention[133,135,146] (Table 4.2, Column 6). In addition, there is emerging evidence to suggest that luteal phase dynamics of progesterone are altered in obesity[70] and PCOS.[154] Lower urinary excretion and a delayed ovulatory rise in PDG have been noted in obese women with regular menstrual cycles,[70] and lower luteal concentrations of progesterone have been documented in women with ovulatory PCOS compared to healthy controls.[154] These findings have implications for the detection of spontaneous ovulation in obese anovulatory patients and suggest that alternative thresholds may be needed to fully capture ovulatory status in PCOS.

Heterogeneity in the Clinical Presentation of PCOS

The dichotomization of the ovulatory response to hypocaloric dietary intervention likely reflects the heterogeneous nature of PCOS. PCOS exists on a spectrum and the diagnosis encompasses a broad range of severity of reproductive and metabolic abnormalities.[2,89] Such differences may impart a variable potential between patients for weight loss to stimulate ovulation. To that end, there is utility in identifying the endocrine or metabolic characteristics that distinguish responders from non-responders prior to dietary intervention. Nine of the studies evaluated baseline clinical predictors of improvements in reproductive function. [125,126,128,130,136,138,140,141,145]

Response was defined in one of two ways across studies: (1) evidence of sporadic ovulation or (2) improved menstrual cyclicity following dietary intervention. None of the studies assessed predictors of the transition to normal ovulatory function. Improvements in menstrual cyclicity were broadly characterized as a decrease in menstrual cycle irregularity, shift from irregular to regular menstrual cycles or shift from anovulatory to ovulatory cycles.[125,126,128,140,141,145] Increased cycle regularity was not necessarily accompanied by improvements in ovulation and a shift from anovulatory to ovulatory cycles appeared to reflect evidence of sporadic, rather than regular, ovulation.[125,141,145] In addition, one study evaluated women with partial or complete recovery from PCOS and identified baseline features associated with the collective normalization of hirsutism, ovulation and menstrual cyclicity.[138]

As shown in Table 4.3, six of the nine studies identified significant clinical predictors of improved reproductive outcomes following dietary intervention.[125,126,128,130,138,145] The baseline characteristics that emerged were largely markers of androgen excess and adiposity. Overall, women with lower circulating concentrations of sex hormone binding globulin,[130] testosterone,[128] androstenedione,[138] and anti-Müllerian hormone (AMH)[125,126,145] were more likely to experience sporadic ovulation and improved menstrual cyclicity during dietary intervention. A lower waist circumference and waist-to-hips ratio were also predictive of recovery from the syndrome.[138] Taken together, these findings suggest that obese women with milder ovarian dysfunction at

baseline may be more likely to experience reproductive benefit from hypocaloric dietary intervention (Table 4.3).

That being said, only three studies directly focused on clinical predictors of ovulation, and of these, two could not identify baseline differences between responders and non-responders.[126,136] Despite their associations with improved menstrual cyclicity, neither androgens nor AMH predicted ovulation following dietary intervention[126,136] (Table 4.3). Inconsistencies in the ability to identify baseline predictors of ovulatory response may have been impacted by two important factors.

First, “response” was broadly defined as an improvement in ovulation or menstrual cyclicity compared to baseline. However, very few studies assessed baseline ovulatory status in their participants (Table 4.2, Column 4). Among the studies that did, serum progesterone or urinary PDG were measured at a single time point[130,138] or on a serial basis for up to two months prior to intervention.[129,140,145] Alternatively, some participants were asked to keep menstrual diaries for one to six months.[127,132,140,141,145,147] While these direct and indirect markers of ovulation were likely used as a reference for improvement, only two of the studies actually published the data that were collected[129,130] (Table 4.2, Column 4). As a result, it was difficult to confirm that the reported changes constituted an improvement compared to baseline and a missed opportunity was noted to characterize variability in the degree of ovulatory response among women. The latter points to some uncertainty that a single baseline characteristic could predict a broad spectrum of reproductive improvements following dietary intervention.

Second, the use of different diagnostic criteria to define study populations resulted in the assessment of ovulatory response across multiple phenotypes (Table 4.1, Column 2). The most commonly accepted criteria for the diagnosis of PCOS (2003 Rotterdam Criteria) identify four distinct clinical phenotypes: (1) Frank (oligomenorrhea, hyperandrogenism and polycystic ovaries), (2) Non-PCO (oligomenorrhea, hyperandrogenism and normal ovaries), (3) Ovulatory (regular menses, hyperandrogenism and polycystic ovaries), and (4) Normoandrogenic or “Mild”

(oligomenorrhea, normal androgen status and polycystic ovaries).[8,9] There is substantial evidence that the severest variants of the condition are Frank and Non-PCO PCOS (phenotypes which are also recognized by the 1990 NIH Criteria).[2,17] Women with combined oligomenorrhea and hyperandrogenism have the most profound disturbances in gonadotropin dynamics, ovarian androgen production and insulin sensitivity, independent of obesity.[11,155,156] By contrast, the other phenotypes seem to represent milder variants of the condition. Women with Ovulatory and Normoandrogenic PCOS have endocrine disturbances that are intermediate to Frank PCOS and healthy controls,[11,155,156] and the presence of metabolic abnormalities seems to depend on the degree of abdominal adiposity.[13] Collectively, these differences suggest that variable improvements in endocrine and metabolic abnormalities may be needed to restore ovulatory cyclicity across phenotypes.

PCOS was diagnosed according to the NIH criteria in seven studies[129,130,134,137,138,141,147] and the Rotterdam criteria in 10 studies (Table 4.1, Column 2). Of the interventions that used the broader definition, three primarily recruited the Frank and Non-PCO phenotypes.[128,135] The inclusion of these two phenotypes implies that the majority of participants had the severest manifestations of the condition and met both the NIH and Rotterdam criteria for PCOS. The remaining studies (*n*=7) evaluated ovulatory response across more heterogeneous cohorts. Of these, four enrolled participants who could be stratified into any of the recognized phenotypes, and as such, included women with evidence of regular ovulation and menstrual cycles at baseline.[127,132,140,146] In some cases, these studies presented outcome data on ovulation for the entire cohort and did not distinguish the women with histories of anovulation from those with normal ovulatory function prior to the intervention[127,132] (Table 4.1, Column 2). The combined assessment of ovulatory status in these distinct cohorts may have masked the independent effect of dietary intervention on ovulation, since improvements would have been challenging to characterize in women with existing ovulatory cyclicity. The other three studies enrolled anovulatory women irrespective of androgen status.[136,139,145] Crosignani and colleagues required

participants to demonstrate combined evidence of chronic anovulation or amenorrhea and polycystic ovarian morphology.[139] The absence of inclusion criteria or data to corroborate androgen excess suggested that the cohort was largely comprised of women with the normoandrogenic phenotype.[139] By contrast, Thomson *et al.* and Kuchenbecker *et al.* screened for hyperandrogenism and the resulting cohorts appeared to include all variants except Ovulatory PCOS[136,145] (Table 4.1, Column 2). It is possible that weight loss would have differential effects on ovarian androgen production in normo- compared to hyperandrogenic phenotypes and that such differences would be difficult to capture using these definitions.

Table 4.3. Baseline endocrine and metabolic characteristics linked to improved reproductive outcomes after hypocaloric dietary intervention in overweight or obese women with PCOS

Outcome	Baseline Characteristic of Responders[a]	Study
Sporadic ovulation	None	Kuchenbecker *et al*., 2011; Nybacka *et al.,* 2013
	Higher SHBG	van Dam *et al*., 2004
Regular (monthly) ovulation	None	None
Improved menstrual cyclicity	None	Moran *et al.,* 2003; Thomson *et al.,* 2008
	Lower AMH	Moran *et al.,* 2007a; Thomson *et al.,* 2009; Nybacka *et al.,* 2013
	Lower testosterone	Nybacka *et al.,* 2011
Recovery from PCOS	Lower androstenedione	Pasquali *et al.,* 2011
	Lower waist circumference and WHR	Pasquali *et al.,* 2011

[a] vs. non-responders. *Abbreviations: SHBG, sex hormone binding globulin; AMH, anti-Müllerian hormone; WHR, waist-to-hips ratio.*

Degree of Change in Salient Endocrine or Metabolic Features Afforded by the Intervention

The dichotomization of ovulatory response to weight loss may also reflect the ability of hypocaloric dietary intervention to recover the endocrine and metabolic abnormalities that impair antral follicle development. Currently, little is known about the salient features or degree of change required to improve reproductive outcomes with weight loss in PCOS.

Ten studies evaluated differences in clinical, endocrine and metabolic characteristics between responders and non-responders at the end of the dietary intervention[125,128,130,134–136,138,140,145] (Table 4.4). Compared to non-responders, women with ovulatory or menstrual improvements after weight loss demonstrated greater reductions in weight or BMI, central adiposity, hirsutism or hyperandrogenemia and indices of insulin resistance.[125,128,130,134–136,138,140,141,145] Changes in gonadotropins and ovarian hormones were similar between groups[130,135,138,140,145] (Table 4.4). Hence, the body of evidence suggests that improvements in weight, adiposity, androgens, and insulin are the primary mediators of restored ovulatory function after hypocaloric dietary intervention in PCOS (Figure 4.1).

Nevertheless, it is prudent to consider that these analyses produced inconsistent results across studies. While the majority of authors noted greater changes among responders, a substantial portion (37–40%) were unable to identify significant differences in weight, hyperandrogenism or insulin sensitivity between groups[125,130,140,145] (Table 4.4). These findings could reflect heterogeneity in the study populations (as described above) or a larger issue surrounding the efficacy of the interventions that were used.

Table 4.4. Studies that have linked changes in clinical, endocrine, and metabolic characteristics to improved reproductive outcomes after hypocaloric dietary intervention in overweight or obese women with PCOS

Outcome	Number of studies that found greater changes in responders[a]	Number of studies that found no differences in responders[a]
Decrease in weight or BMI	5/8 (63%)	3/8 (37%)
Decrease in central adiposity	5/6 (83%)	1/6 (17%)
Decrease in hirsutism or hyperandrogenemia	3/5 (60%)	2/5 (40%)
Improvement in insulin sensitivity	5/8 (63%)	3/8 (37%)
Decrease in mean concentrations of gonadotropins or ovarian hormones[b]	1/4 (25%)	3/4 (75%)

[a] vs. non-responders. [b] Includes luteinizing hormone; follicle stimulating hormone; estradiol; progesterone; anti-Müllerian hormone.

The primary therapeutic targets of caloric restriction are weight and adiposity.[124] In PCOS, it is likely that changes in weight and adiposity stimulate improvements in androgens and insulin, which together precede ovulation and menses.[127] If sufficient weight loss is not achieved, then it follows that improvements in ovulatory function would be unlikely to occur (Figure 4.1). In the studies included for review, women experienced sporadic ovulation with modest changes in weight (i.e. <16%) (Table 4.1 and 4.2). This occurred despite the fact that most women were still classified as obese (BMI ≥30 kg/m^2) at the end of the various dietary interventions.[128,130,134,136,147] A greater degree of weight loss, resulting in a healthier BMI, may be necessary to fully restore ovulatory cyclicity in obese women with PCOS. This idea is supported by preliminary evidence from studies involving bariatric surgery, wherein 100% of anovulatory patients resumed regular ovulation and menses after a 41-kg reduction in weight.[157] However, surgical approaches for weight loss have been associated with significant risks, including post-operative complications, nutritional deficiencies and increased likelihood for deleterious fetal outcomes during pregnancy.[158] Therefore, it is considered wisest to first advocate for dietary and lifestyle modifications in obese women with PCOS.[158] Further studies are needed to determine the optimal degree of weight loss to induce and sustain improvements in ovulatory function, so as to better tailor dietary interventions to individual needs.

In addition, little is known about the time course of caloric restriction that is required to improve ovarian function in obese women with PCOS. Although serial assessments of ovulatory status were performed, the majority of studies did not report the time point(s) of the intervention at which ovulation was observed. Only three studies provided information on this outcome. From these data, it appeared unlikely for an ovarian response to occur within the first two weeks of a dietary intervention.[130,133,141] Specifically, Moran and colleagues did not detect sporadic ovulation until weeks 4–6 or 12–13 of caloric restriction.[141] The time course of these observations may be explained by evidence that endocrine and metabolic responses to caloric restriction precede ovulation.[127,130] Alterations in neuroendocrine feedback were identified in as a few as seven days

on a very low calorie diet,[130] and significant improvements in androgen excess and insulin sensitivity were documented within 2–4 weeks after the onset of dietary intervention.[127] Consequently, a decrease in insulin-mediated androgen production[92] might be expected to stimulate sporadic selection and ovulation several weeks later.[127]

Despite the occurrence of sporadic ovulation, it remains unclear whether short-term caloric restriction is sufficient to normalize ovulatory cyclicity in obese women with PCOS. It has been suggested that turnover of the antral follicles recruited under hyperandrogenic and insulin resistant conditions precedes improvements in ovarian function.[145] Changes in the mechanisms of selection and ovulation may not manifest until a new cohort of follicles is activated from the primordial pool under an improved hormonal environment. Given that a pre-antral follicle takes approximately three months to reach its pre-ovulatory diameter,[33] it is possible that some of the dietary interventions were too short to realize improvements in reproductive function[145] (Table 4.1). Further investigation is needed to determine the precise time course of caloric restriction that is required to normalize ovulatory cyclicity in PCOS.

It is also uncertain whether improvements in ovulatory function can be sustained after a hypocaloric dietary intervention is discontinued. In general, weight maintenance is challenging and weight re-gain is a common problem across populations.[159] This is essential to address in the context of PCOS, as any increase in weight could recover initial endocrine and metabolic disturbances and lead to the rebound of anovulation. Five studies performed follow-up assessments and attempted to capture these changes after the dietary intervention.[127,128,134,138,141] Follow-up assessments were performed on a cross-sectional basis[128,138] or as part of a weight-maintenance intervention that involved regular visits to the research unit.[127,134,141] Ovulatory or menstrual status was largely measured at each time point.[127,128,134,141] Yet, only two of the studies distinguished the data that were collected at the follow-up visits from those collected during the intervention, and menstrual status was the primary reproductive outcome of interest.[128,141] Specifically, Moran and colleagues found that improvements in menstrual cyclicity were sustained

for 4–6 months after caloric restriction in the majority of patients.[141] In some cases, women reported regular menses for an average of two years after the end of the intervention.[128] These outcomes occurred independent of increased energy intake[141] or weight gain.[128,141] Further studies are needed to clarify whether and how these sustained changes in menstrual cyclicity are reflected in the ovaries.

Despite ambiguity surrounding the ideal dietary intervention, current dogma stipulates that caloric restriction is the primary facilitator of weight loss and ovulation in obese women with PCOS.[62,121] Consequently, dietary interventions involving caloric restriction were highlighted in this review. However, it is important to acknowledge that most of the included studies prescribed caloric restriction as part of a larger multifactorial intervention (Table 4.1, Column 5). Emerging data suggest that tailored macronutrient composition, physical activity, and/or behavior modification therapy can augment the effect of caloric restriction on ovulatory function.[121,160] While these additional approaches were not addressed in this review, they have been described extensively by Moran *et al.*[121] and Harrison *et al*.[160] Ultimately, well-designed, randomized controlled trials are still required to determine whether multifactorial dietary interventions can further improve reproductive outcomes in PCOS.[121,160]

CONCLUSION

In summary, commendable progress has been made towards understanding the impact of caloric restriction on ovulatory function in obese women with PCOS. It is clear that modest weight loss is associated with the occurrence of sporadic ovulation in a meaningful proportion of patients and that reductions in hyperandrogenism and insulin resistance likely precede any improvements in reproductive outcomes. The ovulatory response may also depend on the presence of milder reproductive dysfunction at baseline and a greater degree of change in endocrine and metabolic features with intervention. Nevertheless, this review highlighted the variability in the ovulatory response to weight loss and found little evidence to support the effectiveness of hypocaloric dietary intervention to restore normal ovulatory function in PCOS. Because resumption of regular ovulatory cycles may not be a realistic goal for all patients, healthcare providers should be judicious in counseling the degree to which weight loss can be expected to improve ovulatory function in obese patients with PCOS. Future studies would benefit from efforts to improve the accuracy and consistency of measures used to determine and report ovulatory status both at baseline and during dietary intervention. The use of intermittent sampling of biochemical markers, assessment of alternative thresholds for ovulation to better judge luteal function in PCOS, and distinction of ovulation from menstrual cyclicity in reporting improvements is recommended. Finally, the impact of phenotypic variation on the ovulatory response to weight loss should be addressed in order to fully capture the degree and duration of caloric restriction that is needed to effectively promote weight loss and ovulation in PCOS. The identification of optimal dietary and lifestyle approaches to treat anovulation will improve the health and wellbeing of obese women living with PCOS.

SUMMARY AND FUTURE DIRECTIONS

For decades, clinicians and researchers have relied on evidence from histologic studies of folliculogenesis to guide the diagnosis of polycystic ovary syndrome (PCOS) and selection of treatment strategies for anovulation. The notion that follicular excess, arrest, and persistence are universal phenomena in this condition is outdated and needed re-consideration. This work has consolidated the role of ovarian ultrasonography as an effective tool to determine the degree to which antral follicle development is impaired in PCOS. Major findings and implications of this research are summarized below.

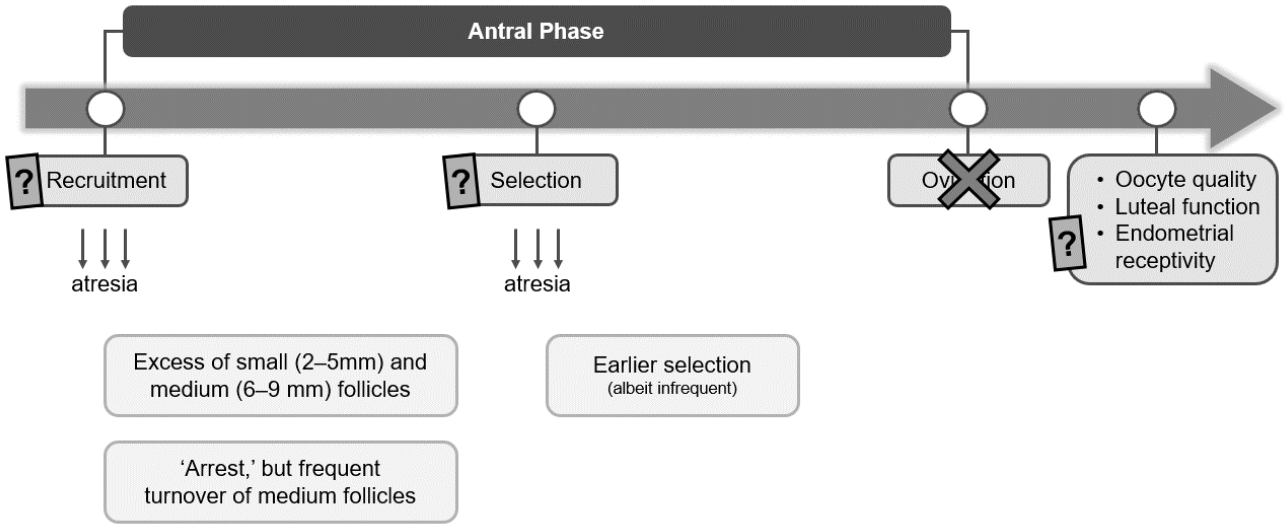
Importantly, we demonstrated the feasibility of capturing antral follicle growth and regression on ultrasonography in women with heightened follicle populations ***(Chapter 1)***. Polycystic ovaries contained more than 100 follicles on any given day of the study. Yet, it was possible to count individual follicles and follow their unique growth trajectories over time. Our approaches were effective[44,75] and consequently hold promise for future evaluations of antral follicle development – both in PCOS and other anovulatory conditions.

We concluded that follicular excess is a constant feature during anovulatory ***(Chapter 1)*** and sporadic ovulatory cycles ***(Chapter 2)*** (Figure S.1). This finding affirms our other recent work, which has shown that sonographic metrics related to follicle number have significant potential to detect PCOS.[66,93] From a clinical perspective, the permanence of follicular excess over time has implications for scheduling diagnostic evaluations of PCOS and reducing undue burden on patients. Our data suggest that menstrual cycle status has little bearing on the ability of follicle number to reflect the condition of PCOS and that random evaluations of ovarian morphology are appropriate ***(Chapter 3)***. Substantial inter-individual, but not intra-cycle, variation in follicular excess ***(Chapter 1 and 3)*** may point to the potential of the ovary to serve as a consistent biomarker of reproductive dysfunction.[88] Importantly, it remains unclear whether follicular excess remains a salient feature across the reproductive lifespan in PCOS. Such an abnormality may

originate in utero or the pubertal transition,[161] but resolve during menopause, as the ovarian reserve depletes.[162] Further studies are needed to understand these changes and whether age-specific diagnostic criteria are needed in this condition.

We confirmed that follicles become “arrested” at the mid-antral stage in polycystic ovaries, but revealed that they also turnover more often than follicles in normal ovaries. This knowledge challenges the traditional theory of follicular persistence and provides a new model of antral follicle development in PCOS ***(Chapter 1)*** (Figure S.1). These data are likely to inform the selection of pharmacologic protocols for anovulation, since exogenous hormones (e.g. recombinant follicle-stimulating hormone, FSH) may accelerate these processes and place women at increased risk for adverse health and pregnancy-related outcomes.

Figure S.1. Proposed new model of impaired antral folliculogenesis in PCOS. *The revised theories for disruptions in recruitment, selection, and ovulation are depicted. Up and down arrows refer to increased and decreased activity, respectively, and “X’s” refer to absent events. Remaining gaps in knowledge are identified with question marks. Follicular graphics were obtained from Ansh Labs.*



We showed that disordered folliculogenesis in PCOS occurs on a spectrum from mild to severe, just like the clinical and biochemical features of the syndrome. Numerous follicles randomly emerge and regress over time and do not demonstrate the same organized patterns of recruitment as in healthy women ***(Chapter 1)***. Follicles can occasionally emerge from this disorder and progress to ovulation, but also exhibit altered growth kinetics, which may signal other disruptions in ovulatory function ***(Chapter 2)***. Together, these studies identified potential defects in follicular recruitment ***(Chapter 1)*** and selection in PCOS ***(Chapter 1)***. Additional analyses, coupled with serial endocrine dynamics, are ongoing to comprehensively capture the extent of impaired folliculogenesis in this condition (Figure S.1).

Ultimately, these data hold promise for understanding the variability in the ovulatory response to hypocaloric dietary intervention in women with PCOS ***(Chapter 4)***. Through our narrative review of the literature, we documented that weight loss is associated with the occurrence of sporadic ovulation – but not ovulatory cyclicity, which is likely the more important outcome measure. However, our assessment of sporadic ovulations in PCOS ***(Chapter 2)*** highlighted a need for further studies to understand the implications of altered growth kinetics on oocyte quality, luteal function, and/or endometrial receptivity. Moreover, our assessment of the available evidence points to a favorable effect of weight loss on ovulation in some (responders), but not all, patients (non-responders). Similar to our findings in ***Chapter 2***, we noted that the ovulatory response may depend on milder reproductive dysfunction at baseline. Future studies, that use accurate methods to detect ovulation, and new knowledge of altered follicular growth in PCOS, are ultimately needed to determine the effectiveness of hypocaloric dietary intervention to normalize the spectrum of disordered antral folliculogenesis in PCOS ***(Chapter 1)***.

Together, these projects provide new insight into the mechanism of anovulation in PCOS (Figure S.1). Our use of ultrasonography enables the immediate translation of our findings into clinical practice to improve the diagnosis and management of women affected by this highly prevalent, broad spectrum endocrine disorder.

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